Abnormalities of HLA and microglobulin expression on tumour cells

Thesis

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Abnormalities of HLA and β_2Microglobulin Expression on Tumour Cells.

A thesis submitted by

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ABSTRACT

This thesis describes the genetic events that can lead to the loss of expression of HLA class 1 on tumour cells. Earlier studies, in colorectal cancer, have shown both complete loss of surface HLA A, B & C expression and loss of single allele products using antibodies recognizing either monomorphic or polymorphic HLA determinants. Data presented demonstrates that complete loss of HLA class 1 expression correlates with mutations in $\beta_2$microglobulin. In a study of 52 colorectal cell lines, 8 showed mutations in $\beta_2$microglobulin leading to loss or reduced HLA expression. In fresh colorectal tumours 9/147 had mutations in $\beta_2$microglobulin, occurring at single or di-nucleotide repeat sequences. From these tumours 71 were analyzed for microsatellite instability, associated with the loss of DNA mismatch repair, and 7 (10%) found to be unstable. $\beta_2$microglobulin mutations were identified in 5 of these tumors. Therefore, mutations in $\beta_2$microglobulin occur more frequently in mismatch repair defective tumours than in colorectal tumours in general ($p<0.01$)

The colorectal cell line HCA-7 lacks expression of HLA-A*0101 based on studies with the polymorphic antibody 142.2, but the normal B cell line (EVA-1224) from the same patient expresses A*0101. This thesis shows that the A*0101 gene, in HCA-7, contains an insertion of a cytidine in a cytidine repeat sequence in exon 4. This mutated A*0101 gene has the similar sequence to that of a rare A*0104'null' allele reported to lack A*0104 expression.

In a model system a $\beta_2$microglobulin-HLA-A*0201 construct was transfected, using an ecdysone inducible expression system, into CHO cells and expression shown to be under the control of the inducer, Ponasterone A. Functional studies demonstrated the construct to be capable of presenting the flu virus peptide GILGFVFTL to a clone of human A*0201 restricted cytotoxic T cells and for the T cells to efficiently lyse the CHO cells.

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STATEMENT

All the work in this thesis was carried out by the candidate except for the following: the HLA class I typing was performed by Susan Tonks in the Cancer and Immunogenetics Laboratory, Oxford; the Cytotoxic T cell assays were conducted with the help of Xiaoning Xu, Molecular Immunology Group, Oxford; and the immunocytochemical analysis of colorectal tumour samples was carried out by Lucus Kaklamanis, Dept. of Pathology, John Radcliffe Hospital, Oxford.

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LIST OF ABBREVIATIONS USED IN THIS THESIS.

A	Adenine residue
ABC	ATP Binding Cassette.
ABI	Applied Biosystems Inc.
ADCC	Antibody-Dependent Cellular Cytotoxicity
AICD	Acivation Induced Cell Death
APAAP	Alkaline Phosphatase Anti-Alkaline Phosphatase complex
ARMS	Amplification Refactory Mutation System
bp	Base Pairs
C	Cytidine residue
CTL's	Cytotoxic T cell Lymphocytes
DMEM	Dulbecco's Modified Eagles Medium
DNA	Deoxyribonucleic Acid
dNTP's	deoxyNucleotide TriPhosphate
DTT	Dithiothreitol
EBV	Epstein-Barr Virus
ECL	Enhanced Chemiluminescence
EDTA	EthyleneDiamine Tetraacetic Acid
ELISA	Enzyme Linked ImmunoSorbent Assay
ER	Endoplasmic Reticulum
FACS	Fluorescence Activated Cell Sorting
FAP	Familial Adenomatous Polyposis
FCS	Foetal Calf Serum
FITC	Fluorescein IsoThioCyanate
G	Guanine residue
G418	Geneticin
γIFN	Gamma-Interferon
HLA	Human Leukocyte Antigen
HIV	Human Immunodeficiency Virus
HNPCC	Hereditary NonPolyposis Colorectal Cancer

IgG	Immunoglobulin G
ILT	Ig-Like Transcript
IPTG	Isopropyl-β-D-ThioGalatopyranoside
ITAM	Immunoreceptor Tyrosine-based Activation Motif
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
IU	International Units
kDa	Kilo Daltons
KIR	Killer-cell Ig-like Receptor
LIR	Leukocyte Ig-like Receptor
LRC	Leukocyte-Receptor Complex
mins	Minutes
mRNA	messenger Ribonucleic Acid
NK	Natural Killer
NP40	Nonidet P-40
PAGE	PolyAcrylamide Gel Electrophoresis
PBS(A)	Posphate Buffered Saline
PCR	Polymerase Chain reaction
PMSF	PhenylMethylSulphonyl Fluoride
RNA	Ribonucleic Acid
rpm	revolutions per minute
RPMI	Rosewell Park Memorial Institute
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulphate
secs	seconds
SSCP	Single Strand Conformational Polymorphism
T	Thymine residue
TAP	Transporter associated with Antigen Processing
TBS	Tris-Buffered Saline
TGF-β	Transforming Growth Factor-β
TNF	Tumour Necrosis Factor
UV	Ultra Violet light
X-gal	5-bromo-4chloro-3-indolyl-β-D-galactopyranoside

CHAPTER 1

INTRODUCTION

1.1.1 The Immune System.

Early work on the immune system indicated that it divided fairly equally into two areas, the mechanisms a body used to produce antibodies to neutralize foreign molecules, called humoral immunity, and the processes involving cell to cell surveillance leading to removal of suspect cells, called cellular immunity. With the aid of modern technology the immune system has now been shown to be extremely complex, but the primarily role is still to defend the body against invasion from many organisms, diseases and foreign molecules. The lymphocytes, which are distributed throughout the body in blood vessels and lymph nodes, are the cells mainly involved in immune responses. These morphologically similar cells have been subdivided according to their expression of surface membrane differentiation molecules. B cells express surface immunoglobulin reactive to native antigens whilst T cells express specialized receptors which recognize foreign molecules, presented at the surface of most cell types in the body, in the form of peptides associated with the HLA system in humans. The T lymphocytes have been further divided into those which express CD8 and recognize small peptides, predominantly from cytoplasmic protein degradation, in association with class I (HLA-A, B & C) molecules and those which express CD4 which recognize larger peptides, frequently from endosomal and lysosomal protein degradation, in association with class II (HLA-DP, DQ & DR) molecules. A further compartment of the immune system has now been described composed of cells derived from a precursor in common with T cells; these are the Natural Killer or NK cells. Unlike T cells, which require stimulation

and clonal expansion to become effective, NK cells express various receptors constitutively and are able to respond immediately by either lysis of infected cells or release of a series of cytokines and chemokines leading to a more general inflammatory response. Many NK cells recognize HLA class I molecules through surface receptors, which inhibit, rather than activate, a response by the NK cells. Cells with reduced or lacking HLA class I expression, such as some tumours and cells infected by certain viruses, may therefore become targets for NK activity.

This thesis concentrates on cellular immunity and in particular on cases where loss of expression of HLA molecules has occurred. The following sections of chapter 1 outline what is currently known about these mechanisms.

1.1.2 History of the HLA system.

The earliest report relating to what has become known as the H2 system in mice was made by Peter Gorer in 1938. He identified a murine red blood cell antigen (antigen II) which segregated in mouse genetic crosses with the acceptance of subsequent tumour transplants (Gorer, 1938). Later the antigen II locus was also linked to a fused tail deformity and had become know as H_2, later to be re-named H-2 (Gorer et al., 1948). About ten years later, Counce *et al.* reported the rejection of tissue grafts between different strains of mice and linked this rejection to a series of histocompatibility loci in the congenic mice strains used and the graft survival (Counce et al., 1956). The genes in the strong (major) histocompatibility locus were reported to have a greater effect on rejection than those genes found at the weaker (minor) histocompatibility locus. The major histocompatibility locus was later shown to be the same as Gorer's antigen II or H-2. Further studies in mice identified three closely linked histocompatibility loci, H2-K, H-2D and H2-L(Klein and Shreffler, 1972; Snell et al., 1971) which encode the mouse major histocompatibility antigens, and many more alleles have been identified at each locus using the terms H2-K^d, H2-K^k and so on.

The major histocompatibility antigens were first reported in humans (now known as HLA, the human equivalent of H2) following the observation that serum samples from multiparous women were capable of agglutinating certain leukocytes. This led to the identification of the LA antigens (Payne et al., 1964) and the 4a and 4b antigens (van Rood, 1962). These became known as HLA-A and HLA-B respectively, and were mapped to the short arm of chromosome 6 (6q21.3) where three closely linked genetic loci were discovered, HLA-A and B and later a third locus C. These loci were seen to be highly polymorphic and by 1987 using serological typing 24 HLA-A, 50 HLA-B and 11 HLA-C alleles had been defined (Bodmer et al., 1987) and with the advent of molecular biological techniques, such as PCR, typing directly from DNA samples has been possible and the current number of alleles identified is 266 HLA-A, 511 HAL-B and 128 HLA-C (EBBL-EBI Database at www.ebi.ac.uk/imgt/hla/stats.html, January 2003). Some of these new alleles are extremely rare and may even be limited to a single individual.

In humans, further genes have been identified on chromosome 6 at 6q21.3 and these form the HLA or MHC region. Historically, these fall into three regions: class I HLA (the highly polymorphic HLA class I alleles), class II HLA (including HLA class II plus TAP, LMP and tapasin genes) and HLA class III (including the complement proteins and cytokines such as TNF).

1.1.3 Structure of HLA class I.

HLA class I is a heterodimeric molecule composed of a highly polymorphic heavy chain glycoprotein of 45 kDa (HLA-A, -B & -C) non-covalently associated with an invariant light chain of 12 kDa (β_2-microglobulin) and an endogenously processed peptide usually of about 9 amino acids in length (Townsend et al., 1989; Wilson and Fremont, 1993). The classical class 1 molecules are generated from three loci on chromosome 6 (HLA-A, -B & -C) whilst β_2-microglobulin is encoded on chromosome 15 (Goodfellow et al., 1975b). They are expressed at the surface of most nucleated cells and have a major role in presentation of peptide

antigens to $CD8^+$ T cells. The non-classical class I HLA molecules are HLA-E, -F & -G which have a similar structure to the classical molecules and associate with β_2-microglobulin but tend to be less widely expressed and at much lower levels. HLA-E appears to have a much more limited repertoire for peptide binding and is involved in NK cell recognition (Braud et al., 1998b)(see section 1.1.6), HLA-G is predominantly expressed on the trophoblast (Kovats et al., 1990; Rinke et al., 1990).

HLA class I genes have 8 exons. Exon 1 encodes a signal peptide, exons 2-4 the three extracellular domains, α1, α2 and α3, exon 5 encodes the hydrophobic transmembrane domain and exons 6-8 encode the cytoplasmic domain (Strachan et al., 1984). With the crystal structure of HLA-A2, Bjorkman *et al.* showed that the membrane distal α1 and α2 domains formed a groove for peptide presentation and that the α3 domain was non-covalently associated with β_2-microglobulin (Bjorkman et al., 1987). Each domain is formed of approximately 100 amino acids and the α1 and α2 domains form two antiparallel α-helices which form the sides of a peptide binding groove with the floor of the groove made up of an eight stranded antiparallel β-sheet, each domain contributing 4 strands. This binding groove is orientated away from the surface membrane, allowing interaction with T cell and NK cell receptors, and is supported by the immunoglobulin-like α3 domain non-covalently linked with β_2-microglobulin. The α1 and α2 domains are highly polymorphic with a majority of the amino acid differences occurring in the antiparallel α-helices. A pocket may be deep or shallow and have charged, polar, neutral or hydrophobic residues, all of which determine the compatibility of the pocket with peptide residues and their side chain characteristics (Matsumura et al., 1992).

The specificity of peptide binding has been shown to be defined by the individual characteristics of the six pockets (A-F) (Colbert et al., 1993; Fruci et al., 1993; Kubo et al., 1994; Tussey et al., 1994). The most important appear to be the A and F pockets, which accommodate the amino- and carboxyl- ends of the peptide respectively. At least one of the other pockets (B, C, D & E) forms an "anchor" to complete the specificity of peptide binding to a particular HLA

heavy chain. The A pocket has three important amino acids contributing to the structure, these are three tyrosine residues at positions try7, try159 and try171. The amino terminus of the peptide is buried into the A pocket leaving the side chain of this first residue pointing upwards into the solvent. There is little preference for any particular amino acid at the amino end of the peptide (Falk et al., 1990). In contrast, the F pocket has distinct preferences for particular residues at the carboxyl end of the peptide. Some F pockets examined are hydrophobic in nature and predominantly bind side chains which are similarly hydrophobic. HLA-A2, for example, has a preference for a peptide with valine or leucine at the carboxyl terminus, where as HLA-B27 has an acidic F pocket that binds basic side chains such as those seen on arginine (Madden et al., 1991). The interactions between the amino- and carboxyl- ends of the peptide and pockets A and F have a stabilizing effect on the HLA/peptide complex and the F pocket initiates some degree of specificity. Some other pockets have a limited repertoire of residues that can be accommodated, these have become known as "anchor" residues. (Barouch et al., 1995).

The binding specificity of the peptide into a groove can be optimized further by water molecules. In a study of HLA-B53, Smith *et al.* have shown that two peptides with widely different P4-P7 residue sequences were equally accommodated in the groove. One peptide, Is6, derived from a malarial parasite protein, had an aspartic acid at P7 with this residue orientated away from the groove leaving a space filled with 7 water molecules. This formed a network of hydrogen bonds stabilizing the peptide backbone in the groove. The second peptide, HIV-2, from the viral gag protein, had a glutamine at P7 which was buried low within the groove causing displacement of the water molecules. Both peptides had an anchor residue of proline at P2 (Smith, 1996).

Only certain residues of the peptide constitute anchor residues and occupy particular pockets in the groove. The remaining pockets can be occupied by a much wider diversity of amino acid residues. This allows for a large range of peptides derived from entirely different protein sources to be expressed by a given HLA molecule. The frequency of specific amino acid sequences, with the relevant anchor residues included, within the general protein pool will also determine the number of possible peptides a particular HLA molecule can bind.

If a particular HLA molecule has a rare order for anchor binding this would limit the number of peptides presented. The order of anchor residues required by HLA-A*0201 are relatively common in proteins and so a large number of peptides can be presented (Falk et al., 1991).

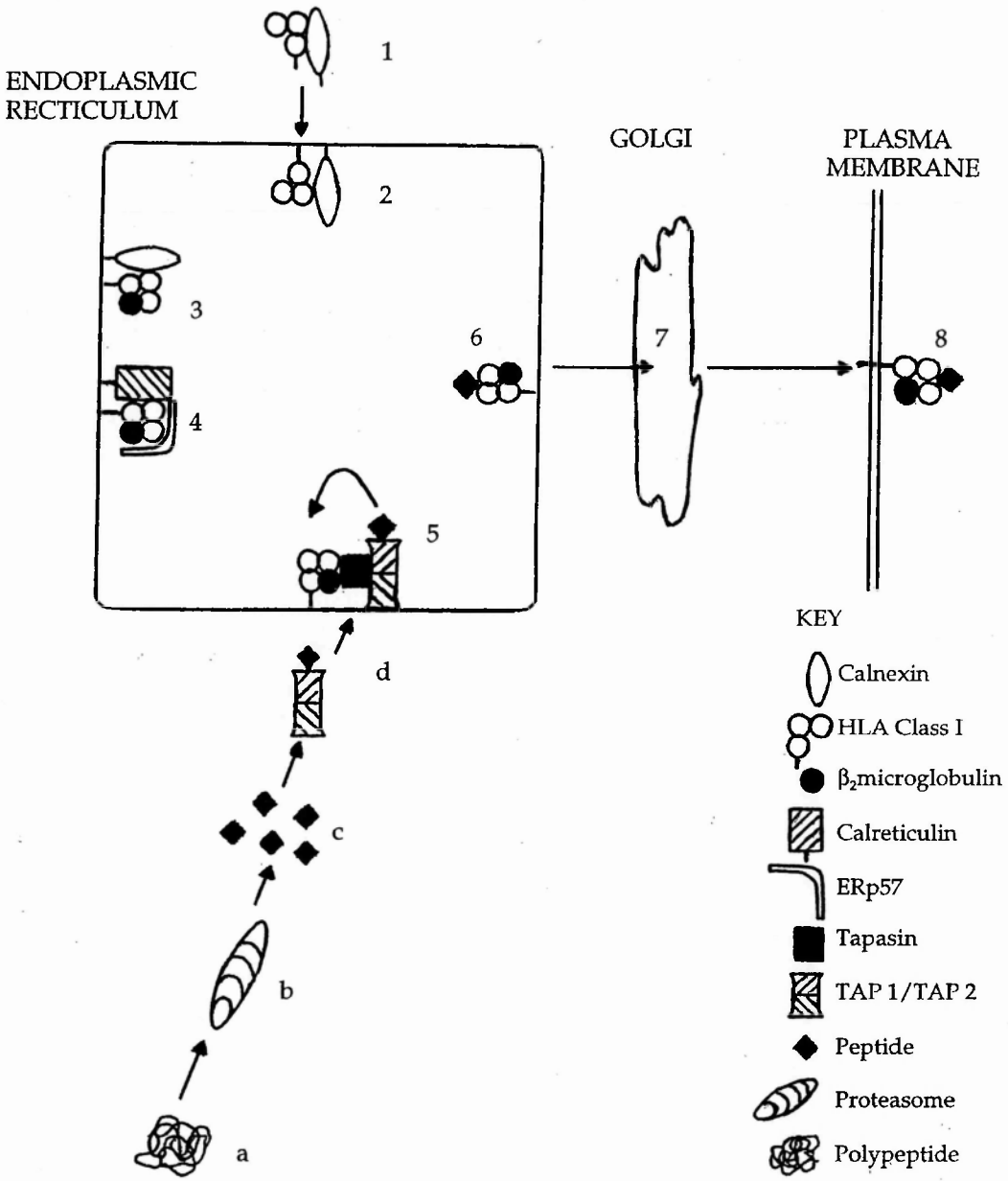
1.1.4 The role of HLA Class I in the peptide presentation pathway.

HLA class I peptide antigen presentation is an intriguing story involving a complex pathway. The pathway is best illustrated by following the progress of a protein, expressed in the cytoplasm, from proteolytic breakdown through to expression on the cell surface membrane. A simplified illustration of this process is shown in Figure 1.

Antigen processing starts with the modification of proteins and polypeptides from the resident protein pool in the cell cytoplasm. This protein pool is made up of endogenous proteins, proteins synthesized in the cell following pathogen infection and "foreign", often functionally impaired, proteins synthesized from altered DNA following a mutational event. These proteins undergo modification into short peptides by proteolysis involving a series of proteasomes. The structure of the 20S proteasome (Baumeister et al., 1998), is cylindrical, consisting of four rings, each made up of seven sub-units. The two outer α sub-units are concerned with maintaining the molecular structure and interacting with proteasome regulatory sub-units, and may also be involved with cellular localization. The two inner β units, forming the centre of the barrel, contain the proteolytic activity (Zwickl et al., 1994). These units are made up of a series of subunits which were initially called LMP-2 and LMP-7, the transcription of which was shown to be inducible by γ IFN (Monaco and McDevitt, 1986). Later a third γIFN inducible sub-unit (LMP-10) was reported together with three further sub-units which are not γIFN induced and may even be down-regulated by γIFN, LMP-9, LMP-17 and LMP-19 (Nandi et al., 1996). The latter are thought to be constitutively expressed. It has been suggested that the β sub-units can be made up of different combinations of the LMP subunits and that this may result in proteasomes that preferentially or more effectively generate peptides capable of

FIGURE 1

A simplified representation of the assembly pathway of HLA Class I molecules



Antigen processing. Polypeptide (a) is cut by the proteasome (b) into small peptides (c) which are transported to the endoplasmic retuculin by the TAP 1/TAP 2 transporter (d).
HLA Class I assembly. Nascent HLA heavy chain binds to the chaperone molecule Calnexin in the cytoplasm (1) and is transported to the endoplasmic reticulin (2). Here $\beta_2$microglobulin binding occurs (3) and Calnexin is replaced by Calreticulin and ERp57 (4) to aid folding and stabilization of the HLA class I/$\beta_2$microglobulin complex. Tapasin then forms a bridge between the HLA class I/$\beta_2$microglobulin complex and the TAP complex carrying peptide (5). Here the peptide is loaded on to the HLA class I/$\beta_2$microglobulin complex. Binding of the peptide triggers disassociation of TAP/Tapasin (6) allowing the HLA class I/$\beta_2$microglobulin/peptide complex to leave the endoplasmic reticulin and enter the Golgi (7) where maturation takes place. Finally, the mature complex is expressed on the surface membrane (8) for presentation of the peptide antigen to CD8 Cytotoxic T cells and NK cells.

high affinity binding to HLA class I molecules. In addition, the preferential incorporation of γIFN inducible sub-units into newly transcribed proteasomes may increase the number of functionally distinct proteasome complexes, generating a larger repertoire of different peptides from a given protein antigen (Gaczynska et al., 1993). As proteins pass through the cylindrical shaped proteasome complex, proteolysis occurs, releasing into the cytoplasm short peptides of between 9 and 23 amino acids. The peptides are then taken up by the TAP complex for transport from the cytoplasm into the endoplasmic reticulum (ER).

TAP is a member of the ABC (ATP-binding cassette) transporters and was discovered about ten years ago. Trowsdale *et al.* reported several new genes in the human MHC class II region, two of which were called RING4 and RING11, which had characteristic CpG island sequences (Trowsdale et al., 1990). Several other groups at about the same time were searching for a candidate gene/s that could be involved in peptide antigen processing and identified in rodents HAM1 and 2 (Monaco et al., 1990) and mtp1 and 2 (Deverson et al., 1990). By 1991 these had all been sequenced and shown to be homologs of two genes which have come to be known as *TAP1* and *TAP2* (Transporter associated with Antigen Processing), the names being accepted by the WHO nomenclature committee (WHO, 1991).

Both *TAP1* and *TAP2* have a similar gene structure with 11 exons and both have an γIFN stimulation response element (ISRE) 198 and 573 bases upstream of exon 1 respectively (Beck et al., 1992). *TAP1* encodes a protein of 748 amino acids while *TAP2* encodes a protein of 686 amino acids. They normally exist as a heterodimer of TAP1 and TAP2 as indicated by immunoprecipitation experiments with a TAP1 specific antibody (Kelly et al., 1992). The *TAP* genes in humans have shown to be polymorphic, with four alleles reported for TAP1 and six for TAP2. The majority of these polymorphisms occur in the transmembrane and nucleotide binding domains, but the exact functional consequences polymorphisms are not known. In an early study of Caucasians, TAP1A had a frequency of 81% and TAP2A 62% (Powis et al., 1993).

The TAP complex (TAP1, TAP2 and peptide) has a fourth component in the form of a 48 kDa glycoprotein which was first reported in co-immunoprecipitation experiments by Ortmann *et al.* (Ortmann et al., 1994). This glycoprotein has been named Tapasin (TAP associated glycoprotein) and is thought to have a bridging role in the association of TAP/peptide complexes with the HLA class I complex, described below. Sadasivan *et al.* have shown, in a series of immunoprecipitation experiments using the β_2-microglobulin negative cell line, Daudi, (which lacks HLA class I complexes) that tapasin is capable of binding to TAP independently of its association with the HLA class I complex (Sadasivan et al., 1996). The exact role of tapasin in the TAP/peptide complex is not known, but it has been proposed that tapasin may act as a tether for the TAP-HLA class I interaction, or it may play a more active role in the uptake of peptides by TAP.

As well as serving as carrier to transport peptides into the ER, the TAP complex also functions as a hub in the ER for newly assembled HLA class I complexes. These complexes have been formed from newly synthesized HLA class I heavy chains assembled with β_2-microglobulin along a pathway involving a series of ER chaperone molecules. Initially, the chaperone calnexin associates with free unfolded class I heavy chains by interactions with both the protein component and monoglycosylated N-linked glycans on the class I molecule. This, however, has been shown not to be an obligatory association as the calnexin negative cell line CEM-NKR is still capable of assembling, and expressing at the cell surface, functional class I molecules (Scott and Dawson, 1995) probably using alternative chaperone molecules such as BiP which has been reported to have functions similar to calnexin (Noessner and Parham, 1995). Partial folding of the class I molecule takes place together with the covalent association with β_2-microglobulin. Once β_2-microglobulin has become involved calnexin is replaced by a second chaperone molecule, calreticulin. The binding of HLA class I/β_2-microglobulin to calreticulin is β_2-microglobulin dependent, however the exact sequence of events is unclear as definitive experiments have proved difficult. Sadasivan *et al.* have suggested that calnexin may interact only briefly with partially folded HLA class I/β_2-microglobulin and is quickly replaced by calreticulin. However, the same workers have shown that the HLA class I will

not bind to calreticulin in the absence of β_2-microglobulin (Sadasivan et al., 1996).

In the sequence of events leading to HLA class I assembly, it is clear that calnexin and calreticulin chaperone the HLA class I heavy chain/β_2-microglobulin complexes on the ER and that tapasin forms a bridge linking the complex to TAP. It has been suggested that the chaperone molecules serve to aid the folding of the HLA class I molecule and together with β_2-microglobulin help to maintain the integrity of the all-important peptide-binding groove of the HLA class I chain. In addition, the chaperones may be involved in the processes leading to degradation of misfolded and unstable empty HLA class I/β_2-microglobulin complexes by marking them for degradation by the proteasomes in the cytoplasm (Wiertz et al., 1996).

Recently, another ER chaperone molecule has been identified by SDS-PAGE following co-immunoprecipitation with a series of antibodies against calnexin, calreticulin, TAP and HLA class I. This chaperone has an apparent molecular weight of 59 kDa and has been identified as ERp57, a molecule with thiol oxidoreductase activity (Hughes and Cresswell, 1998). ERp57 will bind to the HLA class I/β_2-microglobulin complex in a similar manner to calreticulin, in the absence of TAP, but the ERp57 binding to the HLA class I/β_2-microglobulin complex is tapasin dependent (Hughes and Cresswell, 1998; Morrice and Powis, 1998). The role of ERp57 is still speculative, it has been suggested that it may play a part in the folding of the HLA class I heavy chain molecules by controlling and maintaining the disulphide bonds within the heavy chain. ERp57 shares some homology with the protein disulphide isomerase (PDI) (Hirano et al., 1995). Alternatively, ERp57 (also known as ER-60) has been implicated in the process of degrading misfolded glyco-proteins, such as the fully oxidised HLA class I heavy chain/β_2-microglobulin complex, by assisting in reducing and denaturing them in the ER before they are shunted back to the cytoplasm for degradation by the proteolytic machinery (Otsu et al., 1995).

The finally assembled complexes in the ER, which lead to peptide loading, involve the HLA class I heavy chain/β_2-microglobulin/calreticulin/ERp57 complex linked via a tapasin bridge to the TAP/peptide complex. The TAP molecule acts as a hub with up to four HLA class I complexes associated with it, each with a tapasin bridge. It has been proposed that the tapasin bridge interacts with both the TAP1 and TAP 2 moieties of the TAP heterodimer, each interacting with two HLA class I complexes (Androlewicz et al., 1994; Ortmann et al., 1997). Although not proven, there has been a suggestion that the interaction of empty HLA class I molecules with either TAP1 or TAP 2 may enhance the delivery of a particular peptide to an HLA allele expressing a groove with a high specificity for that peptide sequence (Watts and Powis, 1999). There is evidence that if the peptide is longer than preferred, additional trimming can take place within the ER, but it is unclear if this takes place before or after loading into the groove. The specificity of the groove is outlined in section 1.1.3. The transfer of a peptide from TAP into the binding groove of an HLA class I molecule has the effect of stabilizing the HLA class I/β_2-microglobulin and releasing it from TAP. Srivastava *et al.* have reported the presence of a chaperone molecule (gp96) that may protect the peptide from rapid degradation before it is loaded into the empty groove (Srivastava et al., 1994).

Once disassociated from TAP, the stabilized HLA class I/β_2-microglobulin/peptide complex is released from the ER and is transported to the cell surface via the secretory pathway through the golgi apparatus (Brodsky et al., 1996; Williams et al., 1996).

HLA class I/β_2-microglobulin/peptide complexes are expressed on most cell types in the body. There are, however, reports of cells lacking HLA class I expression including spermatozoa (Law and Bodmer, 1978) and placental villous trophoblasts (Goodfellow et al., 1976), whilst other cells such as cortical thymocytes, neurones and hepatocytes express little or no HLA class I (Daar et al., 1984a; Janossy et al., 1980).

Severe down-regulation of HLA class I and/or HLA class II has been described in a comparatively rare number of individuals, most of whom fall into the

classification of type I Bare Lymphocyte Syndrome (BLS). Type II BLS is characterised as demonstrating down-regulation of HLA class II molecules (Touraine, 1978). The type I BLS classification has been further sub-divided into three groups according to the clinical and immunological characteristics:

Group 1. These patients demonstrate loss of HLA class I and β_2-microglobulin at the cell surface, but characteristically have detectable serum levels of HLA class I and β_2-microglobulin. Therefore, these patients are not thought to have alterations in the HLA class I and β_2-microglobulin genes. Heterozygous HLA haplotypes in these patients has also suggested that the gene(s) responsible for the loss of HLA expression are not located in the HLA coding region. Only 4 patients have been reported with type I (group 1) BLS, all of which died of infectious complications within 3 years of life, having suffered from repeated severe bacterial, fungal and parasitic infections from an early age (Schuurman, 1979; Touraine, 1981; Touraine, 1978).

Group 2. These individuals are clinically asymptomatic and the only immunological finding has been the down regulation of cell surface expression of HLA class I and β_2-microglobulin. Decreased levels of mRNA have been reported for both HLA class I and β_2-microglobulin. Only two cases have been recorded, and these are children from the same family. Both children have been HLA typed and were found to have different heterozygous HLA haplotypes (Payne, 1983; Sullivan, 1985).

Group 3. Patients in this group survive into adulthood but are subjected to recurrent bacterial infections and often develop necrotizing granulomatous skin lesions, particularly of the nose and nasal passages. This group, of which there are about 15 reported cases, have reduced or low cell surface expression of HLA class I (Gadola, 2000). Close analysis of 10 of these patients has shown them all to be homozygous at their respective HLA loci and in about half these individuals an underlying defect in the TAP genes has been linked with the reduced levels of HLA class I expression. Examples involving the TAP1 gene include an individual with loss of HLA class I expression exhibiting a homozygous deletion of a G at position 778 in exon 2 of TAP1, leading to a premature stop codon (de la Salle,

1999). A japanese patient has been reported with a homozygous mutation in TAP1, at the slice site between exons 1 and 2. This mutation was identified as a G deletion in codon 200 at the beginning of exon 2 leading to an altered slice site and a frameshift causing a stop codon at codon 228 (Furukawa, 1999). A similar mutation involving the splice site between exons 1 and 2 of TAP 1 was recorded in the fibroblasts of isolated from another patient with BLS (de la Salle, 1999). A mutation in TAP2 has been identified in two children from the same family in Moroco. A brother and sister both exhibit the same homozygous mutation causing a stop codon at codon 253, resulting in a truncated TAP2 leading to a non-functional TAP complex (De La Salle et al., 1994).

1.1.5 The HLA Class II Pathway.

The HLA class II determinants differ from HLA class I in many ways. As already stated in 1.1.1 above, they present larger peptides, frequently from endosomal and lysosomal protein degradation, to the CD4 expressing compartment of the T cell population. HLA class II is a heterodimer of two proteins chains, encoded by the DQ, DP and DR genes, of approximately 30 kD known as α (32 kD) and β (29kD) chains. The difference in size is attributed to N-linked glycosylation. Each chain has two domains (α1, α2 and β1, β2 respectively) with the membrane distal α1 and β1 domains combining to form a single peptide binding groove composed of two antiparallel α–helical loops, the sides, supported by a floor made of eight antiparallel β strands (Ting and Trowsdale, 2002). HLA class II molecules are synthesized *de novo* in the ER where they combine with the invariant (Ii) chain which acts as a chaperone molecule for HLA class II assembly. Ii chains have two portions, one associating with the HLA class II heterodimer, and the other portion, known as CLIP (class II associated Ii peptide), occupies the peptide binding groove (Ghosh et al., 1995). Small peptides present in the ER following proteasome degradation, that usually bind to HLA class I molecules, are prevented, by the presence of the CLIP portion, from binding to HLA class II molecules. The CLIP functioning as a 'stuffer'(Bikoff et al., 1993). The Ii/HLA class II complexes pass from the ER along the secretory pathway into the Golgi and into the *trans*-Golgi network

where they divert and enter the endocytic pathway. From here they accumulate in the lysosomes.

There is still some controversy over how the Ii/HLA class II complexes arrive in the lysosomes. Some favour the idea that the complexes pass straight from the *trans*-Golgi network into the lysosome (Peters et al., 1991). However, Warmerdam *et al.* suggested that the complexes pass to the cell membrane where they are internalised by the endosomes passing into the lysosomes (Warmerdam et al., 1996). Others favour the idea of trafficking from the *trans*-Golgi network via early endosomes into the lysosome (Pond and Watts, 1999). It would seem that the favoured route of Ii/HLA class II complexes depends on the model system and the cell type used, and indeed Hiltbold and Roche have pointed out that utilization of all these trafficking routes by newly synthesized HLA class II molecules would ensure maximum exposure of these molecules to foreign peptides present in the many different intracellular compartments particularly the lysosomal-like antigen-processing compartments or pre-lysosomes (Hiltbold and Roche, 2002).

Having reached the pre-lysosomes the Ii/HLA class II complexes undergo a series of proteolytic enzyme mediated events. First, the Ii is degraded leaving just CLIP occupying the binding groove. This digestion of Ii is mediated by two cysteine protease cathepsins, Cat L and Cat S. At this point two further HLA class II molecules, HLA-DM and HLA-DO associate with the CLIP/HLA class II complex. DM and DO are heterodimers similar in structure to DR, are encoded in the HLA class II region, assemble in the ER and form a pool circulating between the cytoplasm and the pre-lysosomes. Unlike DR, DM and DO show only limited polymorphisms. The role of DM is to assist in the removal of CLIP from the binding groove and the substitution of a peptide from the pre-lysosomal pool. DM is thought to associate with the HLA class II complex to prevent it from being degraded once CLIP has been removed. Although DM is not involved in the peptide binding, it has been suggested that it is able to catalyze the dissociation of sub-optimal peptides, including CLIP, and therefore to have a role in editing peptide binding by selecting the most stable peptide/HLA class II complex associations (Kropshofer et al., 1997; Lanzavecchia et al., 1992).

The function of DO appears to be a still a matter for debate. DO is known to require an association with DM to enable it to leave the ER and very little DM has been identified without DO associated (Alfonso and Karlsson, 2000). Several experiments indicated that the presence or absence of DO influenced the number of HLA class II molecules expressed at the cell surface with CLIP occupying the binding groove (Denzin et al., 1997).

The mode of antigen internalization is diverse and can include such processes as macropinocytosis, phagocytosis and receptor-mediated uptake, but these processes are closely regulated. The uptake depends on both the type of antigen and the type of antigen presenting cell involved and these will dictate the endocytic compartment to which the antigen will be targeted (Lanzavecchia, 1996). Once an antigen has entered the antigen presenting cell it travels along the endocytic pathway and encounters en route a series of enzymes capable of processing the antigen. It is not completely destroyed, but is broken down into polypeptides of variable length that are able to bind into the HLA class II groove. Initial breakdown takes place at low pH with the activity of GLIT (γIFN-inducible lysomal thiol reductase) which catalyses the reduction of disulphide bonds (Aruncchalam et al., 2000). This unfolds the antigen allowing access by other proteolytic enzymes such as cysteine, aspartic and serine proteases and metalloproteases. These proteases make up the cathepsin enzymes of which Cat L and Cat S have already been mentioned in connection with Ii digestion. There are at least 7 known Cat enzymes which are synthesized in the ER as pro-enzymes which become active in the endocytic pathway with the aid other proteases (Riese and Chapman, 2000; Turk et al., 2001; Watts, 1997). Once Cat activity has started, early binding to HLA class II may occur, this protects the permissive peptide sequence that will bind in the groove leaving the overlapping ends to be trimmed at a later stage (Villadangos and Ploegh, 2000; Watts et al., 1998).

The peptide/HLA class II complex leaves the endosome and becomes expressed on the cell membrane. The route of trafficking is not properly understood. It has been suggested that the lysosomes fuse directly with the cell membrane (Wubbolts et al., 1996), alternatively, small vesicles, derived from the endosomes,

may carry the peptide/HLA class II complex to the cell membrane for presentation to effector cells (Amigorena et al., 1994).

1.1.6 Antigen presenting cells.

HLA class I/β_2-microglobulin/peptide complexes are expressed on most cell types in the body with the exception of certain cells such as spermatozoa and placental villous trophocytes mentioned above in section 1.1.4. The distribution of HLA class II expression is, however, more restricted than HLA class I. Normally, HLA class II is found on B lymphocytes and some macrophages, and on certain specialized antigen presenting cells such as dendritic cells in lymphoid tissues, Langerhans cells in the skin and on epithelial cells found in the thymus (Daar et al., 1984b; Janossy et al., 1980). Many cell types that do not normally express HLA class II molecules can be induced to express them following stimulation with lymphokines such as γIFN, although the mechanism of antigen presentation after γIFN simulation is not clear (Basham and Merigan, 1983).

Probably the most important antigen presenting cells are the Dendritic Cells (DC). These are bone marrow derived cells which represent a heterogeneous family of cells with different functional capacities. DC's are found throughout the body, but are concentrated in areas of potential antigen entry, especially near epithelial and mucosal surfaces, where they capture and process exogenous antigens. In addition, DC's are found in the thymus, where they have a role in establishing tolerance to self-reactive antigens, and in secondary lymphoid organs, where they present antigens to specific T cells (Jefford et al., 2001). Resting steady state DC's present in blood and other non-lymphoid tissues reside there as immature cells with morphologically distinct cytoplasmic extensions that are short and veil-like, and demonstrate a weak capacity to stimulate naive T cells. Disruption of this steady state during inflammation triggers the release of mediators that induce both the maturation and migration of DC's. During this process DC's translocate HLA/peptide complexes to the cell surface, increase expression of T-cell costimulatory molecules such as CD40, CD80, CD86 (and activation marker CD 83 in humans) and the intercellular adhesion molecules

(CD54 and CD58) involved in cell/cell interactions between DC's and T cells. In addition, the morphology of DC's changes with lengthening of the cytoplasmic extensions (Nijman et al., 1995). Mature DC's express the chemokine receptor CCR7 and migrate through the lymphatic vessels to T cell areas of lymph organs in response to chemotactic gradients of the CCR7 ligands CCL19 and 21 (Morelli and Thomson, 2003).

In humans and in mice DC's are quite rare cells, and without a lineage specific marker having been identified, have been difficult cells to study in detail. However, multiple subsets of DC's have been identified, based mainly on the expression of a series of cell surface markers, and also on their functional characteristics. The major subsets in humans are the $CD11c^+$ myeloid and the $CD11c^-$ plasmacytoid DC's. The equivalent $CD11c^+$ myeloid DC's in mice express myeloid markers such as CD11b, 33D1 and adhesion related F4/80 (Wilson and O'Neill, 2003). These cells are found in the marginal zones of lymph nodes and spleen but migrate to the T cell areas of the spleen upon stimulation. They are efficient stimulators of $CD4^+$ and $CD8^+$ T cells and show effective MHC class II presentation to antigen-specific $CD4^+$ T cells (Pooley et al., 2001). Plasmacytoid $CD11c^-$ DC's differ, and in humans express high levels of CD68, CD36, BDCA-2, IL-3α (IL-3-recepter α chain) and ILT3 (an inhibitory receptor) (Salio et al., 2003). Morphologically these cells very rarely show the cytoplasmic extensions and they are thought to enter the lymph nodes directly from the blood via the high endothelial venules, possibly aided by the expression of the chemokine receptor CXCR3 and CD62L (L-selectin). Upon activation these DC's produce large amounts of IFN type I (Cella et al., 1999). It has been found in mice that plasmacytoid DC's express low levels of MHC class II and that this set of DC's may play a role in the maintenance of peripheral tolerance, however, in humans mature plasmacytoid DC's are capable of inducing proliferation of naive $CD4^+$ T cells and more recently, in an *in vitro* system, to efficiently present an endogenous antigen, via the HLA class I pathway, to an HLA class I restricted $CD8^+$ T cell clone (Salio et al., 2003; Wilson and O'Neill, 2003).

It has generally been accepted that peptides derived from viral and other intracellular infectious agents are presented on the cell surface by HLA class I to $CD8^+$ cytotoxic T cells. Exogenous proteins, on the other hand, are processed via phago-lysozomes and replace the invarient clip fragment associated with HLA class II for presentation to $CD4^+$(Th) cells and usually involve specialized antigen presenting cells such as DC's. However, there has long been evidence that exogenous antigens are capable of being presented to $CD8^+$ cytotoxic T cells via HLA class I expression on certain DC's (Heath and Carbone, 2001). This has become known as cross-priming or cross-presentation of exogenous antigens which has been defined as the presentation of exogenous antigen via the HLA class I pathway for the generation of stimulatory or tolerogenic responses in $CD8^+$ cytotoxic T cells (Zinkernagel, 2002). This concept was based on the idea that activation of $CD4^+$(Th) cells or $CD8^+$ cytotoxic T cells requires antigen (signal 1) plus costimulation (signal 2) whilst encounters involving signal 1 alone result in anergy or deletion.

Identification of the subsets of DC's capable of either priming or inducing tolerance in vivo has provided evidence on whether the same or different DC subsets initiate tolerance or priming. A CD8 ($CD205^+$) DC subset has been shown to cross-present antigens derived from ovalbumin-loaded spenocytes and induce priming of $CD8^+$ cytotoxic T cells (den Haan et al., 2000). This same DC subset appears to be responsible for the cross-presentation of pancreatic auto-antigens to induce cross-tolerance (Belz et al., 2002). As the same DC subset seems capable of eliciting both outcomes, it is likely that a quiescent DC constitutively sampling self-antigens in the periphery is tolerogenic and requires further specific stimuli to convert it into a mature DC able to deliver all the signals necessary for $CD8^+$ T cell priming (Bonifaz et al., 2002). In the absence of inflammatory signals, most DC's in vivo will be functionally immature and are likely to induce tolerance.

The generation of cytotoxic T cells against tumour antigens may occur either by the direct recognition of antigens on tumour cells, or by cross-presentation of tumour antigens involving professional antigen presenting cells such as DC's. The major route of tumour antigen presentation is still controversial. Several

studies support cross-presentation as the predominant mechanism (Huang et al., 1994; Wolfers et al., 2001), whilst other workers suggested cross-presentation may not contribute to the generation of cytotoxic T cells (Kundig et al., 1995; Ochsenbein et al., 2001). These type of studies have been acknowledged as difficult because of the limited number of DC's available to study and with problems involving the efficiency of HLA class I presentation on tumours and it has been suggested that tumour progression may occur despite efficient cross-presentation of tumour antigens and generation of antigen-specific $CD8^+$ cytotoxic T cells (Nelson et al., 2001).

Some understanding of DC involvement in tumour antigen presentation has come from the numerous attempts to treat cancer patients by immunotherapy using DC's. Also more efficient methods have been established for the isolation of DC's and for manipulating them in culture, allowing preparation of both immature and mature DC's. These cells have then been loaded with different sources of tumour associated antigens. Vaccination using DC's peptide-pulsed, with a peptide derived from a known tumour associated antigen, has been shown to induce both peptide-specific $CD8^+$ and $CD4^+$ T cells in healthy volunteers and in melanoma patients (Dhodapkar et al., 2000; Schuler-Thurner et al., 2002). Exosomes, formed by the fusion of the plasma membrane with multivesicular endosomes and contain various adhesion and costimulatory molecules and heat shock proteins, are secreted by DC's and tumour cells. Tumour-specific cyotoxic T cells have been activated with DC's loaded with exosomes derived from tumour cells and, in mice, exosomes derived from peptide-pulsed DC's have been shown to induce anti-tumour responses (Wolfers et al., 2001; Zitvogel et al., 1998).

Several speculative rationales have been proposed which could improve the efficiency of DC-based immunotherapy. Infecting mature DC's with recombinant viruses containing the cDNA from a given tumour antigen is one possible approach. In a model system, the EBNA-3A peptide, derived from Epstein-Barr virus, and introduced into mature DC's, was efficiently presented inducing antigen-specific cytotoxic T cells in vitro (Subklewe et al., 1999). To circumvent the problems associated with viral vectors, Smith et al. introduced into mature

DC's a plasmid containing the full length DNA of a melanoma associated polyepitope and were able to demonstrate the induction of multiple cytotoxic T cell responses (Smith et al., 2001). Another proposal was to let DC's phagocytose whole tumour cells (which were dead or dying) and tumour cellular fragments, with the intention of inducing cross-presentation of tumour antigens on both HLA class I and II molecules, allowing the simultaneous induction of tumour specific cytotoxic T cells and $CD4^+$ helper cells (Mandruzzato et al., 1997; Toes et al., 1999). But it has been pointed out that such a strategy bears the risk of inducing autoimmunity, as the DC's could present self-peptides as well as tumour-specific antigens, however, Steinman et al. have suggested that immature DC's will induce tolerance to self-antigens derived from apoptotic cells (Steinman et al., 2000).

1.1.7 The Effector cells: Cyotoxic T Lymphocytes and NK cells.

The effector cells can be divided roughly into three compartments, (i) CD8 expressing cytotoxic T lymphocytes (CTL's), (ii) Natural Killer (NK) cells and (iii) CD4 expressing T cells mostly not cytotoxic. The $CD8^+$ CTL's and certain receptors found on NK cells interact with HLA class I/peptide complexes and the $CD4^+$ CTL's recognize longer peptides in association with MHC class II molecules.

(i) Naive $CD8^+$ CTL's are triggered to proliferate and enter a differentiation pathway when their T cell receptors (TCR) bind to self-HLA class I/peptide complexes expressed on a virus-infected cell. This peptide presentation is usually performed by HLA-A and B (Young and Uhrberg, 2002). Upon activation, the $CD8^+$ CTL's express CD56 and the inhibitory receptor Ig-like transcript 2 (ILT2)/leukocyte Ig-like receptor 1 (LIR1), a receptor belonging to the leukocyte-receptor complex (LRC) which is a polymorphic cluster of related genes found on Human chromosome 19, at 19q13.4, and encodes a series of Ig super-family protein receptors. ILT2/LIR1 has been shown to recognize a wide range of HLA class 1 molecules (Young et al., 2001). In addition, during activation the CTL

begins the synthesis of a set of cytokine molecules, perforin and granzymes that become localized into secretory granules. A majority of these activated CTL's enter the effector pool and when they recognize further virally infected cells are able to release the granules onto the surface of the infected cell. The perforin forms pores in the membrane allowing the granzymes to enter and initiate a complex pathway leading to apoptotic cell death through the direct cleavage of pro-caspase-3 or indirectly through caspase-8 (Barry and Bleackley, 2002). A majority of the activated CTL's are destined to die through activation-induced cell death (AICD), but a small proportion survive to become memory T cells (Marrack et al., 2000). The precise events, which influence an activated $CD8^+$ CTL to become a memory T cell, are still controversial. One hypothesis suggests that the cells deleted by AICD express only the ILT2/LIR1 receptor from the LRC, whilst those that go on to become memory cells additionally express one of the killer-cell Ig-like receptors (KIRs), that are also encoded in the LRC, and acquires resistance to apoptopic cell death by increased expression of Bcl-2 (Grayson et al., 2000; Young et al., 2001).

(ii) Natural killer (NK) cells represent approximately 15% of the circulating lymphocytes and were originally named because of their ability to kill in a non-specific manner (Kiessling et al., 1975). NK cells have a large granular morphology and are able to lyse certain cells in the absence of stimulation (Trinchieri, 1989). In the bone marrow NK cells share a common precursor with T cells, which is distinct from that giving rise to B cells. NK cells are derived from $CD34^+$, $IL15R^+$ cells in response to IL15 released from the bone marrow stromal cell population. During this development a sub-set of NK cells acquire CD16 expression. CD16 is a transmembrane-anchored 70 kD glycoprotein of the Ig superfamily also expressed on activated monocytes and a subset of T cells (Daeron, 1997). It is a low affinity Fcγ III receptor, expressed in association with FcεRI-γ which binds to IgG coated target cells and signals through an immunoreceptor tyrosine-based activation motif (ITAM), located in the cytoplasmic tail of FcεRI-γ (Aramburu et al., 1995; Galandrini et al., 1996). On CD16 mediated-activation, the $CD16^+$ sub-set of NK cells release of a series of cytokines and lyse target cells by antibody-dependent cellular cytotoxicity (ADCC) (Perussia et al., 1984).

During IL15 induced development, NK cells also acquire the expression of CD56 (Cooper et al., 2001) together with a number of receptors from both the LRC Ig superfamily, also present on some $CD8^+$ T cells, and the C-type lectin superfamily. The members from the LRC Ig superfamily include the KIR and ILT receptors, which are structurally similar transmembrane molecules, classified by their structure. For example KIR-2D, the predominant type in humans, has two extracellular Ig domains and KIR-3D has three extracellular Ig domains. Both KIR-2D and KIR–3D can be sub-classified according to their cytoplasmic structure as -long, containing one or more of the ITIM (I/VxYxxL/V) inhibitory sequences, or -short, lacking the ITIM, but exploiting a membrane-bound, homodimeric adapter molecule (DAP12) that contains an ITAM (immunoreceptor tyrosine-based activation motif) sequence that activates NK activity (Lanier et al., 1998; Natarajan et al., 2002). Thus, based on their structure, KIR's and ILT's when binding to their ligands can either activate or inhibit NK activity. In a recent review by Vilches and Parham over 100 sequence variations have been reported for the KIR family and these have been divided into 13 groups according to their structure (Vilches and Parham, 2002). The ligands for KIR's and ILT's have been shown to be the HLA class I molecules, but in only a few cases has the specificity been identified. Most work has been done with inhibitory KIR's (KIR-2DL2 and –2DL3) that recognize HLA-C alleles.

Members of the C-type lectin superfamily of receptors also become expressed during IL15 induced development of NK cells. Most members of this family are heterodimeric, composed of a 30kDa glycoprotein invariant chain, CD94, linked by disulphide bonds to one of the 43kDa glycoprotein receptors, NKG2. These are all closely linked genes on human chromosome 12p12.3-p13.1 at the 'NK gene complex' (Yabe et al., 1993). CD94 is a single gene with very limited allelic polymorphisms, whilst the NKG2 family is composed of four genes, *NKG2A, -C, -E* and *-D*, with a splice variant of *NKG2A* known as NKG2B. The extracellular, ligand-binding domains of NKG2A, -B, -C and -E share a high degree of homology, but the cytoplasmic regions are different. NKG2A and -B have long domains, with two ITIM inhibitory motifs in their tails, whilst NKG2C and -E have short tails that do not signal directly, but associate with the signaling

molecule KARAP12/DAP12 which contains a dimeric ITAM activating component (Burshtyn et al., 1997; Selvakumar et al., 1996). NKG2D has only 20%-30% sequence identity with the other NKG2 family members and is expressed as a homodimer associated with the adapter molecule, DAP10, through which it triggers an activating signal (Cifone et al., 1997). Early studies on the ligands for the CD94/NKG2 indicated recognition of HLA-A, -B, -C, and -G, based on cytotoxicity experiments using anti-CD94 and anti-CD94/NKG2 antibodies (Phillips et al., 1996; Soderstrom et al., 1997). More recent work on possible ligands for CD94/NKG2A have shown it to recognize HLA-E that is expressing peptides derived from the leader sequences of other HLA heavy chains (Borrego et al., 1998). In detailed studies using tetrameric complexes of HLA-E, only transfectants expressing CD94/NKG2A, -B, or -C stained positively (Braud et al., 1998a). The NKG2D homodimer recognizes MICA and MICB, which are non-classical MHC-1 homologs, composed of α1, α2 and α3 domains but do not associate with β_2-microglobulin and do not bind peptides. MICA and MICB are minimally expressed on normal tissue and are upregulated on stressed cells and in epithelial tumours (Groh et al., 1999).

NK cells have been sub-divided according to their expression of CD16 and CD56. A majority (approximately 90%) of cells express high levels of CD16 (the Fcγ III receptor) with low or absent expression of CD56 whilst the minority of NK cells show low or even lack expression of CD16 but have high expression of CD56. These two subsets of NK cells also have differing expression of other NK receptors. The CD56 (high) cells express little KIR and ILT2 and have high expression of CD94/NKG2A. In contrast the CD56 (low) cells express high levels of KIR and ILT2 but CD94/NKG2A is low or absent (Cooper et al., 2001).

Activation of NK-cell cytotoxicity is brought about by a balance of inhibitory and activatory signals mediated by the NK cell receptor repertoire with additional information from various adhesion and costimulatory molecules (Leibson, 1997). Resting CD56 (low) NK cells appear to be more naturally cytotoxic, but after IL2 activation both subsets have similar levels of cytotoxicity. The CD56 (high) subset are the major source of γIFN produced by NK cells, whilst the more granular CD56 (low) NK cells exhibit greater levels of ADCC (Nagler et al., 1989).

(iii) $CD4^+$T cells, which recognize larger peptides presented via the HLA class II molecules, initiate a series of events that lead to the proliferation of the antigen-peptide presenting cells. Naive T helper cells become activated by their first contact with peptide/HLA class II complexes in the lymph nodes leading to the secretion of a series of cytokines including IL-1, 2, 3, 4, 5, 10 and γIFN. The IL-1 signals local dendritic cells to leave the tissue areas and migrate into the lymph vessels where they mature and present ingested antigen, at the cell surface, as peptide/HLA class II complexes, together with newly synthesized B7 molecules (Jenkins et al., 2001). After recognition by antigen-specific CD4 T cells, naive T cells secrete high levels of IL-2 and undergo rapid proliferation in an IL-2 independent manner, in response to an unidentified T cell growth factor (Khoruts et al., 1998). During this proliferation stage, IL-12, produced in response to adjuvant activity in the antigen-presenting dendritic cells, and γIFN, from NK cell sources, then induce differentiation of the T cells into Th-1, or inflammatory T cells (Kearney et al., 1994; Scott, 1993). Many of these cells die in the T cell areas, but some survive in response to the anti-apoptotic effect of adjuvants on dendritic cell IL-12 production. Of the T cell survivors, those that divided the most and received the highest concentrations of IL-12 lose chemokine receptor 7 (CCR7) expression, gain the P-selectin ligand and have the capacity to produce γIFN and other cytokines which activate macrophages. This increases their ability to kill phagocytosed microorganisms, and to recruit macrophages, lymphocytes and neutrophils to the site of activation. Those T cells that received lower concentrations of IL-12 retain CCR7 and acquire rapid IL-2 producing potential, but lack the ability to produce γIFN. The $CCR7^+$ cells are able to re-circulate through the lymph tissues whilst the $CCR7^-$ cells are excluded from the lymph areas and circulate in the blood and tissues that express P-selectin. If antigen is cleared most of the $CCR7^-$ cells die but small numbers can revert to the $CCR7^+$ status and become part of the T memory pool (Jenkins et al., 2001; Travers, 2000).

In the early stages of differentiation, IL-12 leads to the polarization of naive T cells into Th-1 cells. It has been suggested that at this time the presence of γIFN has little effect on the developing Th-1 cells, but may inhibit their differentiation

into Th-2 cells (Constant and Bottomly, 1997). When the IL-12 level decreases, IL-4, secreted by basophils, mast cells and NK1.1$^+$ CD4 cells, can induce naive T cells to differentiation into Th-2 cells. The Th-2 cells produce a cocktail of cytokines including IL-4, 5, 6 and 10 which have the effect of activating B cell differentiation into antibody producing cells (Constant and Bottomly, 1997; Travers, 2000).

1.1.8 Immunosurveillance.

The idea that tumours must "escape" from immune recognition contains the assumption that immune responses are capable of destroying tumour cells, however, without immunological pressure there would be no requirement for tumours to escape immunosurveillance. The immunosurveillance hypothesis dates back to the 1950's when Burnet suggested that the immune system of a host could recognize antigens of newly arising tumours and eliminate these tumours before they became clinically evident (Burnet, 1957; Burnet, 1967). Progressive cancer was thought to be a rare event, only seen when the tumour cell escaped the efficient control of the immune system. More recent ideas claim that the immune system dose not play this central role in controlling early tumour development, however, the contribution of the host immune system and the relevance of T and B cells in combating tumour growth are still subject to debate (Pardoll, 2001; Smyth et al., 2001).

Several studies have demonstrated that the immune system is capable of mounting a response to tumour cells during the early stages of tumour development. Two studies involving NK cells and γδ T cells have examined their potential roles in immunosurveillance. The first study explored antitumour immune responses elicited by tumours transduced with the NKG2D ligands, retinoic acid early transcripts 1b (Rae-1b) or H-60 and found that NK and/or CD8$^+$ T cells mediated potent antitunour immunity (Diefenbach et al., 2001). The study used NKG2D ligand-transduced cell lines that express high amounts of Rae-1b of H-60. Although this study demonstrated a possible mechanism for immunosurveillance involving NK cells, it was pointed out that NKG2D

expression on spontaneous tumours would probably be much lower than in the experimental system. A second study has reported a possible role for NKG2D$^+$ γδ T cells at the early stage of tumour development (Girardi and al, 2001). Both these studies were carried out in mice using comparatively short time scales, and high concentrations of antigen that could alert the immune system to respond, and it has been questioned how relevant these finds are to the human situation where tumours may take years to develop, usually with a low antigen expression (Khong and Restifo, 2002).

The development of the immune system may well have been to protect an individual against infection from pathogens and viruses, and the immune system coevolved with infectious diseases in a way that allows the survival of the host and the pathogen. Protection from these diseases is particularly important in young individuals both from the point of view of the survival of the individual and the survival of the species at large. Cancers, on the other hand, usually arise in individuals beyond the reproductive age and are more of a threat to the individual rather than the population as a whole. Thus, the immune system did not develop special strategies to combat tumours, but instead has to deal with tumours using mechanisms initially shaped to protect an individual from infectious pathogens and viruses.

The specific immunological mechanisms activated for the efficient removal of a bacterial or viral infection are dependent on the route of entry used by the infectious agent. Haematogenously spread infections such as *Streptococcus pneumonia, Haemophilus influenzae,* enteroviridae, measles, and poxvirus are controlled by neutralizing antibodies of the IgM and IgG classes and protect the vital organs (Baumgarth et al., 2000; Fenner et al., 1974). In contrast, noncytopathic viruses, for example hepatitis B and C, that infect the peripheral solid organs are largely controlled by activated T lymphocytes which have the capacity to extravasate and enter the infected tissue to lyse infected cells (Zinkernagel et al., 1996). Solid tumours, which frequently express cell-associated tumour antigens, appear to the immune system to resemble viral infections of peripheral tissues and may elicit a similar response to that used to protect against

a noncytopathic virus. This response would predominately be the activation of cytotoxic T lymphocytes (Ochsenbein, 2002).

At least two ideas have been put forward as to how tumours are able to grow. One model suggests that the successful growth of a tumour happens when the tumour "escapes" from the early mechanisms of immunosurveillance (such as described above) and that as the tumour develops, in the presence of constant surveillance, it progressively "shapes" it's immunological phenotype to avoid immune recognition. A second model suggests that established tumours are able to grow without immune-mediated rejection because they appear to the immune system to be normal cells. In this model the cells are thought to resemble normal healthy cells that do not send out danger signals to activate an immune response because they express neither microbial immune-recognition patterns nor releases distress signals to alarm the innate immune cells (Restifo and al, 2002). During progressive growth, a tumour may become more immune-activating. This can be the result of a number of factors such as damage or disruption to surrounding cells, triggering of stress responses caused by outstripping of nutrients and oxygen supplies which may lead to pH imbalances, generation of reactive oxygen radicals, up regulation of stress protective factors (such as heat shock proteins) and death by necrosis or apoptosis. In addition, as the tumour grows progressively, dysregulated genetic and epigenetic events will lead to the potential expression of increasing numbers of neoantigens (Khong and Restifo, 2002).

Both the humoral and cellular components of the immune system are capable of mounting a response to tumour antigens. During most CTL antitumour responses production of tumour specific antibodies has also been seen. These antibodies are not toxic to the tumour cell *per se*, but act by blocking vital signaling molecules on the cell surface or by a secondary effector mechanism including ADCC (involving NK cells) and complement-dependent cytotoxicity (Carter, 2001). In mice, antimelanoma antibodies inhibit tumour growth in a FcγR-dependent manner (Clynes et al., 1998) and clinically important antibodies in humans, transtuzumab (Herceptin) and rituximab (Mabtera, Rituxan), have also been shown to function via a Fc- FcγR interaction (Clynes et al., 2000).

Lymphocytes (T and B cells) and the professional antigen presenting cells (such as macrophages and DC's) have been implicated in contributing towards an immune response to tumour antigens. These cells tend not to function as individual cells, but interact and collaborate in organized lymphoid tissues such the follicles, marginal zones, germinal centres, and red pulp associated with lymph nodes and spleen. These anatomical structures determine the localization of antigen, cytokines, and bystander contacts through costimulatory molecules (Zinkernagel et al., 1997). The time scale taken and the amount of viral or tumour antigen that reaches the secondary lymphoid organs is crucial for the induction of CTL's. Antigens that never reach these organs in sufficient amounts are ignored by the immune system. Some viruses have developed strategies for avoiding immune recognition such as the Papilloma virus that infects the basal layers of epidermal cells and replicates only in keratinocytes without infecting Langerhan's cells, hence staying outside the lymphoid tissues (Frazer, 1992; Ochsenbein et al., 2001). Tumours tend to arise from a single cell in the periphery, outside the secondary lymphoid organs, and early expansion of the tumour results in either no or very few of the tumour cells reaching the secondary lymphoid organs. In a study of various solid tumour cells, transfected and expressing a glycoprotein from the lymphocytic choriomeningitis virus (LCMV-GP), tumours were either transplanted peripherally into mice as solid fragments or injected subcutaneously (s.c.) as a single cell suspension. The solid fragments grew well outside the lymphoid organs and no tumour cells were detected in the lymph nodes and no LCMV-GP specific CTL's were induced. In contrast, the single cells injected s.c. reached the lymph nodes and antigen specific CTL's were identified. The same study demonstrated that the peripheral solid tumours did not tolerize the mouse immune system, because when replicating LCMV or antigen-expressing DC's were introduced to the animals, a tumour specific CTL response was detected. In addition, suspensions of tumour cells introduced s.c. into mice already bearing a peripheral solid tumour load mounted a tumour specific response indicating that the initial solid tumour had not tolerized the mouse (Ochsenbein et al., 1999; Ochsenbein et al., 2001). These experiments indicate that the immune system can be ignorant of the presence of small early solid tumours but that early trapping of tumour cell in the secondary lymphoid tissues can be beneficial in eliciting an immune response. However, the detection of tumour cells in the secondary lymphoid organs of patients

usually indicates a worsening situation, and the fact that tumour cells are detectable in the secondary lymphoid organs suggests the tumour cell must have evaded recognition by the immune system to have migrated to these organs.

The CTL response is usually to antigens that become transiently presented in the organized lymphoid organs and they do not react to antigens continuously present. Indeed, continuously present antigens can actively delete T cells specific for that antigen by T cell exhaustion. Some noncytopathic infections have been reported to delete T cells by this mechanism and an overwhelming infectious dose of a rapidly replicating virus can also cause this form of tolerance (Moskophidis et al., 1993). The development of lymphohaematopoietic tumours that infiltrate the secondary lymphoid organs should induce an efficient immune response. The fact that this could happen may explain the increase incidence of these type of tumours in immunosuppressed patients (Kelly et al., 1998).

T cell activation has been shown to require two distinct signals. The first signal is the interaction of the T cell receptors with HLA class I molecules expressing peptide antigens. The second signal is a more nonspecific signal provided by costimulatory molecules such as the interaction of CD28 on T cells with members of the B7 family of molecules on professional antigen presenting cells, and members of the TNFR family (CD 40 and CD27) as well as soluble molecules, for example IL-2 and IL-12. T cell receptor stimulation alone, without a second signal leads to T cell anergy or deletion (Matzinger, 1994; Schwartz, 1990; Townsend and Allison, 1993). For efficient activation of T cells, the costimulatory molecules are required to be present, and to be of sufficient concentration, especially in the secondary lymphoid organs. Tumour antigen and second signal-expressing tumours when introduced into lymph node deficient aly/aly mice failed to induce a specific T cell response. The same result was seen if the cells were injected as single cells or as solid tumour fragments. In these mice, which lack secondary lymphoid organs, the immune system ignored the tumour cells (Ochsenbein et al., 2001). Some observers claim that tumours expressing costimulatory molecules are usually rejected more efficiently than control, non-costimulatory molecule expressing, tumours. However, these studies do not distinguish between induction and enhanced maintenance of effector T cells (Maric et al., 1998; Townsend and Allison, 1993).

Tumour cells can produce a variety of cytokines and chemikines that can have a negative effect on the maturation and function of an immune response. For example, tumours of the lung, head and neck as well as breast cancers frequently secrete vascular endothelial growth factor (VEGF). *In vitro* studies show VEGF inhibits the differentiation and maturation of DC's through the suppression of the transcription factor NF-κB in haematopoietic stem cells (Oyama and al., 1998). An inverse correlation has been reported in gastric cancers between VEGF expression and density of DC's within the tumour, a finding that has been associated with a poor prognosis (Saito et al., 1998). An elevated serum concentration of IL-10 is also frequently seen in cancer patients. IL-10 can exert an inhibitory effect on DC differentiation and maturation from stem cell precursors and has been reported to inhibit IL-12 production and induction of T helper type 1 responses (Sharma and al., 1999). In addition IL-10 enhances DC apoptosis (Ludewig and al., 1995) and may protect tumour cells by down-regulating HLA class I (by down regulation of TAP1 and TAP2), HLA class II and ICAM-1 expression (Salazar-Onfray and al., 1997; Yue and al, 1997).

Many tumours have been reported to use varying strategies to evade recognition by the immune system, for example, down regulation of HLA class I (see section 1.2.6), antigen loss, antigen modulation, and the expression of molecules that inhibit the immune response (Chenn et al., 1998; Garrido et al., 1997; Johnson et al., 1998). However, various antigenic tumours have been reported to successfully grow in immunocompetent hosts, including melanomas, renal cancers and breast cancers, and in some of these cases tumour infiltrating lymphocytes have been identified (Whiteside, 1994) indicating evidence of an immune response, but one unable to eradicate the tumour. Patients who are immunosuppressed have been reported to be at a higher risk of developing virally triggered tumours and tumours of lymphohaematopoietic or vascular origin, but these individuals are not thought to show an increased incidence of the more common tumours of the breast, colon, lung (Kelly et al., 1998).

Whatever the role/s of immunosurveillance are, it is usually accepted that once a tumour is clinically detectable, spontaneous regression is very rare for a vast majority of tumour types. Whether tumours need to escape immunosurveillance

is not clear from current knowledge. Also, there is limited evidence that the immune system is capable of initiating a response to tumours and that the responses seen are capable of causing tumour destruction (Restifo and al, 2002). Some claims contradict the idea that the immune system spontaneously mounts a lethal attack on tumour cells and that cycles of immune activity contribute to the shaping of a tumour phenotype, allowing it to progressively escape such immune responses. However, established solid tumours generally do not have growth spurts, they tend to grow, and keep growing bigger (Khong and Restifo, 2002).

1.1.9. Tumour Antigens

60 years ago. research using mice showed that immune responses could lead to the rejection of a sarcoma transplanted between genetically homogeneous C3H mice without toxicity to the normal tissues in the animals (Gross, 1943). However, in humans, it was not until 1989 that the first naturally occuring 'tumour specific antigens' were identified that could elicit a cytotoxic T cell response to an autologous melanoma (Knuth et al., 1989). Since then, many tumour antigens have been identified and by targeting them meaningful clinical responses have been seen.

Tumour antigens can be broadly speaking divided into four different categories: i) Tumour antigens consisting of proteins that are expressed only in the normal adult tissue counterparts of cancer cells, and are defined as differentiation antigens. ii) Antigens generally not detectable in normal adult tissues and expressed by different tumours of the same histology. iii) Antigens not expressed by normal tissues, but detectable on a wide variety of histologically unrelated tumours. iv) Tumour antigens in the form of epitopes generated by mutated proteins; in most cases these tumour antigens are individual antigens uniquely expressed by the tumour bearing the particular mutation (Renkvist et al., 2001).

The identification of specific tumour antigens has been made using several different strategies. The 'biochemical' strategy has been to acid-elute peptides

bound to HLA class I molecules from tumour cells, followed by fractionation of the peptides with reverse-phase high performance liquid chromatography. The resulting peptide fractions were then assayed for their ability to sensitize HLA class I matched target cells for lysis by tumour specific cytotoxic T cells. With this approach yeilds are small, but in several cases sufficient material has been isolated to allow sequencing of the peptide, hence allowing a search of various data bases to identify the gene/s encoding the antigenic peptide (Cox et al., 1994).

The 'genetic' strategy has been employed in several forms. One approach has been to isolate cytotoxic T cell clones with specificities for autologous tumour cells, then attempt to identify by cDNA expression cloning, the gene/s, within the tumour cells, that code for the T cell–recogized epitopes. Again this approach has mainly been used to identify HLA class I restricted epitopes, but a recent report has indicated that similar methods can be used to identify HLA class II epitopes (van der Bruggen et al., 1991; Wang et al., 1999). A second 'genetic' stratergy has been the development of the SEREX technique whereby an expression library of potential tumour antigens, expressed as a λ-phage library, is screened using serum taken from cancer patients. This method assumes that the serum will contain antibodies directed to recognize specific tumour antigens (Chen et al., 1998). In the future, more tumour antigens may be identified using DNA mico-array technology to search for genes that are over-expressed, however, this approach will not measure the immunogenicity of a potential antigen and will require careful choice of control/normal material.

The third strategy for identifying tumour antigens is the 'reverse immunology' method. This approach is the 'reverse' of the previous strategies in that the initial observation has been the identification of a protein that is mutated or over-expressed on a given tumour cell. Peptides, derived from the sequence of the identified protein, are then selected for by high binding affinity to a specific HLA class I molecule, and are loaded onto antigen-presenting cells to stimulate lymphocytes *in vitro*. Demonstration that the peptide is a tumour antigen is acheived by showing that the stimulated cytotoxic T cells are able to kill the given tumours cells expressing the putative tumour antigen presented by matched HLA class I molecules (Smith and Cerundolo, 2001).

A considerable number of tumour antigens have now been reported. In the examples given below all have been demonstrated to elicit T cell responses and in many cases the peptide sequences identified that are capable of priming CLT's for these responses. Where known the presenting HLA class is indicated.

MAGE – This represents a family of over 20 tumour antigens first identified in melanoma (Lucas et al., 2000; van den Eynde et al., 1995). These genes encode the cancer-testis antigens. Peptides from MAGE 1 are presented by HLA-A1 and MAGE 3 by both HLA –A1 and –B44 (Nestle et al., 1998). Other members of this family include BAGE (Boel et al., 1995) and GAGE (De Backer et al., 1999).

PRAME – Preferentially Expressed Antigen of Melanoma was first isolated as a human melanoma antigen recognized by CTL's. The expression has now been reported on normal testis and on various solid tumours and on a series of leukaemias including AML. CML, ALL, lymphoma and multiple myeloma. Five epitopes have been identified which have been used to prime CTL responses, these have been associated with HLA-A*0201 and –A*2402 (Matsushita et al., 2003).

NY-ESO-1 – This is another cancer-testis antigen which has high expresssion on a number of different tumours including breast (30%), prostate (25%), ovarian (25%) and melanoma (45%) (Chen et al., 1997). This antigen has been used in immunotherapy studies where patients, with metastatic disease, have received injections of three HLA-A2 binding peptides derived from NY-EOS-1. Some of the patients in the study were later shown to have peptide specfic $CD8^+$ T-cell responses and several individuals demonstrated stabilization of disease with some regression of individual metastases (Jager et al., 2000).

gp100 – Glycoprotein-100 is a differentiation antigen expressed on melanomas and also expressed on normal melanocytes. Several HLA-A2 binding peptides including (gp100 209-217) and (gp100 280-289) have been successfully used to induce CTL responses in patients with metastatic melanoma. However, it has been pointed out that these immune responses may affect normal melanocytes (Salgaller et al., 1996).

MUC-1 – Human epithelial mucin is a large polymorphic transmembrane glycoprotein that is expressed on most simple epithelia. The extracellular domain

is composed of 20 amino-acids tandom repeats with highly *O*-glycosylated regions rich in serine, threonine and proline (Gendler et al., 1990). Over-expression of MUC-1 has been reported in breast, lung, pancreatic, colon and ovarian tumours where it is usually seen as having shorter, more heavily sialylated carbohydrate side chains when compared to MUC-1 expression on tissues of similar origin (Gendler and Spicer, 1995). Two HLA-A2 restricted peptides have been identified, one in the terminal repeat domain (M1.1) and a second in the leader sequence (M1.2) (Brossart et al., 1999).

CEA – Carcinoembryonic antigen is a large (200 kDa) protein and is a differentiation antigen expessed on a sub-set of normal epithelial tissues and has high expression on tumours of the colon, pancreas, breast and lung and their metastases. CEA is released from tumour cells into the circulation and has been used in post-operative surveillance for the presence of residual tumour cells (Macdonald, 1999; Thompson, 1995). One peptide has been identified, CEA CAP1 (codons 571-579) which is presented on HLA–A2. This peptide has been used in immunotherapy studies where patients immunized with CEA CAP1 (or a derivative, CEA CAP1-6D, in which the sixth amino acid, asparagine, has been replaced by a glutamic acid) have been shown to develop reactive CTL's both by CTL assay and by tetramer staining (Fong et al., 2001).

TGFβRII- Transforming Growth Factor β receptor type II is expressed in normal epithelial cells, but the expression of a mutated form has been reported in certain colorectal cancers where the mutated form is only expressed in the tumour cells. About 90% of these colorectal tumours type as microsatellite unstable, associated with the RER phenotype, and demonstrate a mutation in the from a single adenosine deletion in a poly(A)tract located in the coding sequence of the TGFβRII gene. This frame shift mutation creates a peptide sequence capable of stimulating T cells, via *in vitro* presentation by HLA-DR, which later demonstrated antigen-specific T cell responses (Saeterdal et al., 2001). Interestingly, the HLA-DR restricted sequence encompasses a smaller peptide sequence which can be naturally processed by HLA-A2 and able to prime, in healthy donors, a good antigen-specific T cell response (Linnebacher et al., 2001)

SART-3 – This is a potential colorectal tumour specific antigen. Expression has been reported on a number of colorectal tumours, and on normal testis and foetal liver. The only records of expression on other normal cells are on cultured fibroblasts and PHA stimulated T cells. In a study by Ito et al. three epitopes

drived from SART-3 were used to stimulate uncloned CTL's and later used to monitor tumour cell lysis. Killing was resricted to allogeneic colorectal carcinoma cell lines expressing HLA-A2 (Ito et al., 2000).

EpCAM – EpCAM is another differentiation antigen identified many years ago using antibody AUA-1. Immunohistochemical analysis indicated reactivity in colonic epithelial cells within colon tumours, but no rectaivity with normal mature gastric mucosa (Makin, 1985). Since then Nagorsen et al. have reported an HLA-A2 restricted epitope, codons 263-271, capable of prime T-cells from patients with metastatic disease, and demonstrated a T cell response in *in vitro* CTL assays (Nagorsen et al., 2000).

Her-2/neu (also known as c-erb-B2) – This belongs to the family of epithelial growth factor receptors that have been shown to be over-expressed on 30% of breast and ovarian carcinomas and on a sub-set of colorectal tumours. It is widely expessed on normal epithelial tissues, including breast, in human fetuses and adults (King et al., 1985). One study, using a peptide consisting of codons 654-662 was presented by HLA-A2 to uncloned T cells from patients with metastatic disease. CTL responses were then shown against Her-2/neu positive tumour cells expressing the same HLA class I (Nagorsen et al., 2000). However, in earlier studies, the stability of the Her-2/neu peptides had been questioned when little or no CTL responses had been identified. To address this a cocktail of three 15 aa peptides had been used to serially immunize patients with breast cancer. These peptides covered different regions of the gene previously suggested as tumour specific epitope sites. Presence of CTL activity was demonstrated by lysis of Her-2/neu positive tumour cells. In these studies the CTL activity could still be detected one year later with both $CD4^+$ and $CD8^+$ cell responses (Fisk et al., 1995; Rongcun et al., 1999).

K-ras – K-ras is a proto-oncogene which has been shown to be mutated and over-expressed in a number of different tumour types, and in colorectal cancer up to 50% of tumours and cell lines, derived from carcinomas of the colon, carry a K-ras mutation. Interestingly, almost all the mutations occur in codons 12 and 13. The sequence of theses two codons are: GGT GGC, and it appears that the third base in each coldon is particularly prone to a point mutation. Two studies have looked at the immunogenicity of each of these codons. Abrams et al. isolated T cells from a HLA-A2 patient with a colon carcinoma carrying a mutation in

codon 12 of K-ras. Pror to isolation of the T cells the patients had been immunized with peptides consisting of codons 5-14 of a mutated K-ras, with different aa's at codon 12. Stable T cell responses were demonstrated for both $CD4^+$ and $CD8^+$ clones which reacted with colon cancer derived cell lines carrying the same codon 12 mutation to the patient (Abrams et al., 1997). Fossum et al. on the other hand isolated $CD8^+$ T cells from a patient carry a colon cancer expressing a (Gly13->Asp) mutation of K-ras. These T cells were maintained and primed with a long (25) peptide of codons 1-25 of K-ras, including the codon 13 mutation. On CTL analysis, using colon cancer cell lines (including examples carrying a (Gly13->Asp) mutation), showed cell lysis in a HLA-B44 restricted manor (Fossum et al., 1995).

Most of the above examples of tumour specific antigens have been analyzed to some depth and specific epitopes identified that lead to immunoresponses such as isolation and priming of T cell clones. In a vast majority of cases the presentation of these epitopes has been by HLA class I molecules, especially A locus molecules, particular HLA-A2. However, evidence is now accumulating to show that occasionally B locus molecules are involved in presentation of tumour specific antigens and more recently increasing evidence shows that C locus has an important role in tumour antigen presentation.

T cell lines obtained from melanoma-infiltrated or noninfiltrated lymph nodes, from a patient who had remained disease free after 8 years post surgery for melanoma, were demonstrated to elicit T cell responses to autologous melanoma cells. These effector cells were directed against the tyrosinase-related protein-2 (TRP-2) and gp100 melanoma epitopes and shown to be presented in an HLA-Cw8 restricted manner. Further investigation using COS cells transfected with HLA-C*0802 identified specific peptides presented by HLA-C*0802 for both TRP-2 and gp100. These peptides were used to prime peripheral blood derived T cell clones from the melanoma patient and shown to efficiently lyse autologous melanoma cells in CTL assays (Castelli et al., 1999).

In an attempt to identify peptides presented by HLA-Cw4, Buchsbaum et al. used a method involving the transfection of cells with expression vectors to

encode a truncated form of HLA lacking the transmembrane domain. These transfected cells secrete a soluble form of HLA (sHLA) into the growth medium, thus allowing accumulation of large amounts of sHLA with expressed peptides. Separation of the resulting peptides by capillary reversed-phase HPLC and analysis by tandem mass-spectrometry identified over 54 different peptides presented by HLA-Cw4 from two transfected cell lines, MDA-231, a breast cell line, and UCI-107, an ovarian cell line (Buchsbaum et al., 2003). Using sequence databases proteins were identified that contained the peptide sequences. The majority of the peptides were derived from normal proteins, but several were related to tumour progression. HLA-Cw4 expressed on both cell lines peptides derived from FAS (Fatty Acid Synthase), Putative Prostate Tumour suppressor and Epithelial Cell Transforming oncogene. The breast cell line expressed peptides from Mucin-5B, Topoisomerase I and Rac 1b protein whilst only the ovarian cell line expressed ART1 (Adenocarcinoma antigen). A number of these genes have been reported to be abnormally expressed in tumour cells including ART-1 (Nishizaka et al., 2000), FAS (Kuhajda et al., 2000), Topoisomerase-I (Lynch et al., 2001) and Mucin-5B (Perrais et al., 2001).

Panelli et al have demonstrated that HLA-Cw*0702 is involved in the presentation of peptide 170-178 derived from the melanoma antigen MAGE-12. Experiments were performed using tumour infiltrating lymphocytes (TIL's) obtained from the same needle biopsy that lead to the establishment of the melanoma cell line, F001. Initial data indicated that the restriction element could have been either HLA B*0702 or Cw*0702. However, using human embryonic kidney (293) cells transfected with an HLA-Cw*0702 construct, they were able to screen a cDNA library derived from the F001 tumour cells. A 100-gene pool was identified and subsequent cloning of the pool lead to identifying a sequence homologous to MAGE-12. Synthetic peptides of various MAGE-12 sequences, predicted to bind to HLA-Cw*0702, were screened using the TIL's in a IFN-γ stimulation assay and peptide 170-178 elicited the strongest response (Panelli et al., 2000).

Tumour antigens have now become the focus of attention because they form the basis of the development of anti-cancer vaccines and immunotherapy. Fundamental to these deveolpments is the assumption that tumours differ from

normal tissues by tumour antigens which are either unique, tumour specfic antigens (TSA), or relatively restricted to tumour tissue, tumour associated antigens (TAA). As a consequence of the presence of these TSA's or TAA's the tumour is capable of inducing a specific immune response in the form of a complex of integrated actions of a variety of immune cells (Begent, 1999; Herlyn and Birebent, 1999). However, as described above, tumour cells have developed many mechanisms to escape immunesurveillance. Against this background different strategies have been used for the development of experimental immunotherapy to cancer cells, the two main approaches being to elicit an effective anti-tumour immune response either by potentiation of the immune response or by modifying the tumour cell to become more immunogenic (Ada, 1999; Ward and al., 2002). These strategies can take the form of 'active specific' and 'passive' immunization strategies.

The 'active specific' approach is to prime the naive immune T cells *in vivo* by presenting tumour antigens via antigen presenting cells, in the context of HLA along with the necessary costimulatory molecules. This has been achieved using intact irradiated tumour cells, gene-modified tumour cells, viral oncolysates, tumour peptides conjugated to an immunogenic carrier or administered in combination with an immune adjuvant and recombinant viral vectors containing the tumour antigen encoding gene (Ockert et al., 1999). A variation of these techniques has been to use *ex vivo* loaded professional antigen presenting cells such as DC's (Wong et al., 1998). Antibodies can also mediate the activity of various active, non-specfic effector systems. Tumour specfic monoclonal antibodies can mediate cytolysis either by engaging NK cells (the $CD16^+$ sub-set expressing the Fcγ III receptor) lysing tumour cells by ADCC or by complement activation. Antibodies used in this way have now been altered or 'humanized' to reduce human anti-mouse reponses such as Herceptin (Trastuzumab) introduced to treat metastatic breast cancer (Huls et al., 1999; Vogel and al., 2001).

In 'passive' strategies, immune system components are added systemically or directly at the site of the tumour. This type of adoptive immunotherapy can be performed by taking a patient's autologous immune effector cells and enriching them for a sub-population of anti-tumour immune cells, by sorting and

expanding *ex vivo* the anti-tumor effector cells of interest are re-introduced to the patient (Morse and Lyerly, 1999). Cytokines such as TNF-α, given either alone or fused to anti-tumour antibodies, have been shown to induce tumour regression (Ockert et al., 1999).

Increasing numbers of tumour specific antigens are being reported and they have lead to the development of many cancer vaccine studies. In a recent review of cancer vaccines, Finn (Finn, 2003) has observed a move away from vaccines based on well-defined tumour antigens, due to dissatisfaction with the results obtained in the clinic, in favour of vaccines using whole tumour cells or whole cell lysates as antigens. These complex antigen preparations include unique tumour antigens expressed only on an individual tumour that, by analogy to unique tumour antigens seen in mouse carcinogen-induced tumours might be more immunogenic and promote a strong immune response (Srivastava, 2000). Experiments performed in mice transgenic for tumour antigens expressed by many tumours, but not usually expressed on normal tissues, have shown such antigens can elicit a strong immune response and even tumour rejection (Gendler and Mukherjee, 2001; Greiner et al., 2002). Finn argues that vaccines based on defined tumour antigens should not be under-estamated as they have been successfully used in animal models in which they have been tested almost exclusively in tumour prevention, but the poor results in humans reflects the use of these vaccines as therapeutic agents in advanced disease, often after the failure of other treatments (Finn, 2003).

1.2.1 COLORECTAL CANCER.

In recent years a considerable weight of literature has accumulated reporting and discussing the genetic alterations that underlie the histological and morphological evolution of colorectal tumours. With more sensitive techniques available it is now possible to screen gene by gene for potential mutations and epigenetic changes that may influence the change of cells from normal to malignant, and to gain an insight into the functional characteristics that mutations might confer on protein-protein and protein-DNA interactions leading

to the malignant phenotype. This introduction will be confined to a basic review of carcinogenesis relating to colorectal cancer, with a bias towards the possible role of HLA in tumour progression.

1.2.2 Epidemiology of Colorectal Cancer.

Following lung cancer in men and breast cancer in women, colon cancer is the next most common cancer in the western world leading to a cancer-related death. In England and Wales approximately 30,000 new cases are diagnosed each year, with about 17,000 actual deaths per year (HMSO, 1989; HMSO, 1992). The cases do not seem to demonstrate a gender bias and fewer than 20% of the cases occur before the age of 50 (Safford et al., 1981). Sites for tumour growth can be anywhere along the large bowel, but appear to be more common in the caecum/ascending colon (38%) and the sigmoid colon/rectum (35%).

Most (95%) of these cases are sporadic occurring in individuals with no observed family history of colon cancer. About 5% of cases arise in patients with an inherited predisposition; the best characterized being familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC). Epidemiological studies suggest that environmental factors can have a major influence on the incidence rate of colorectal cancer. This has been based on the observation that in the 'western world' colorectal cancer occurs at about ten times the rate than that in regions such as Eastern Europe, South America, Asia and Africa (Waterhouse et al., 1982). Furthermore, migrants from a low risk area, such as Japan, that live long term in a higher risk area, like the USA, show an increase rate of colorectal cancer incidence (Haenszel and Kurihara, 1968). Influences on these increases in incidence have been attributed to geographical environment, diet and nutritional factors. Red meat, animal fat, refined carbohydrates and alcohol (Giovannucci et al., 1995b; Giovannucci et al., 1994b; Willett et al., 1990), in addition to smoking (Giovannucci et al., 1994a) are associated with an increase in risk, whilst a diet high in vegetables, fruit, vitamins (particularly A, C and E) and physical exercise (Giovannucci et al.,

1995a; Giovannucci and Willett, 1994) have been suggested as protective or associated with a lower incidence.

1.2.3 The Normal Large Bowel.

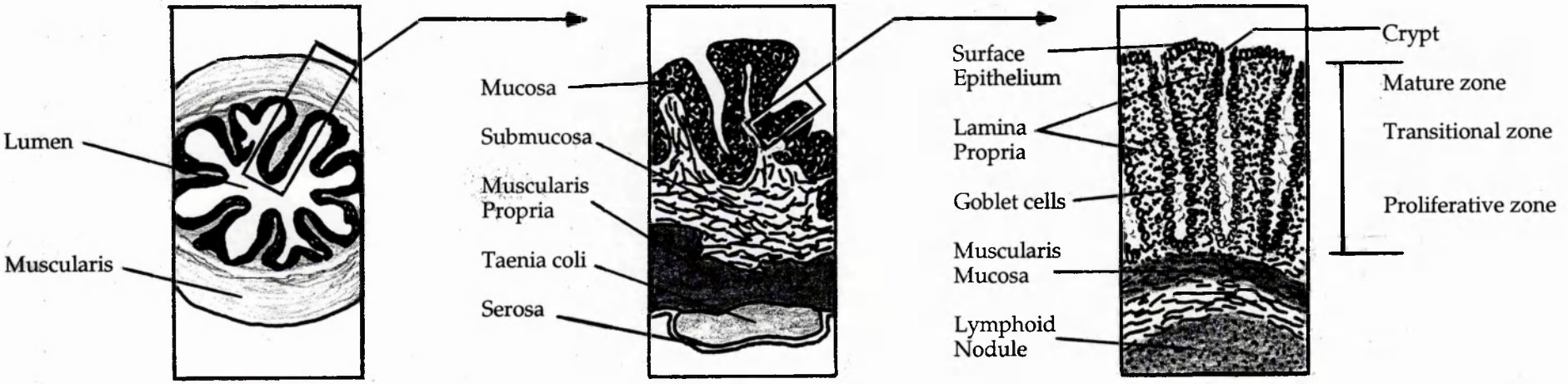
Anatomically, the last 1 to 1.5 metres of the gastrointestinal tract form the large bowel which can be sub-divided into six regions: caecum, ascending colon (on the right side), transverse colon, descending colon (on the left side), sigmoid colon and rectum. The rectum forms the distal region of about 10 cm and ends at the anal canal and anus. The large bowel is formed of four cellular layers: mucosa (the inner most layer composed of epithelium, lamina propria and muscularis mucosa), submucosa, muscularis propria and serosa (see Figure 2).

The mucosal surface is covered by a single layer of low columnar to cuboidal epithelium into which regular spaced crypts of LieberKuhn open, these crypts extend down into the muscularis mucosa layer (Burkitt et al., 1995). The mucosal epithelium is composed of two cell types: (i) the absorptive enterocyte cells, which are involved in the cellular transport of water and electrolytes in addition to having a secretory function, and (ii) the goblet mucous cells which secrete mucin and other high molecular weight glycoproteins. The rectal mucosa contains a higher number of goblet cells compared to the large bowel and at the recto-anal junction the mucosa abruptly stops being replaced by stratified squamous epithelium. In the crypts, the epithelium contains both the mature absorptive enterocytes and goblet cells plus immature and undifferentiated precursor stem cells and endocrine cells (Potten and Loeffler, 1990).

The lamina propria contains a network of connective tissue, collagen fibres, smooth muscle bundles, lymphatics, blood vessels and nerves, with occasional lymphocytes, plasma and mast cells. The crypts that penetrate into this layer are surrounded by a sheath formed of fibroblasts and myofibroblasts (Richman et al., 1987). These cells synthesize extracellular matrix proteins that contribute to the basement membrane, including collagens and fibronectins. The muscularis

FIGURE 2

AN ILLUSTRATION OF THE NORMAL LARGE BOWEL AND ITS HISTOLGICAL STRUCTURE



mucosae, a thin layer of smooth muscle, separates the lamina propria from the submucosa layer.

The colonic epithelium undergoes a continual process of cellular renewal with a rapid turnover every 3 days. Cellular replication occurs in the middle (the proliferative zone) of the crypts with high rates of cell mitosis and DNA and RNA synthesis. New immature cells proliferate and migrate upwards towards the top of the crypt, but by the time they reach the transitional zone, proliferation ceases and DNA and RNA synthesis decreases. The process of maturation begins and is completed as the cells move up into the top zone, the maturation zone. In this zone the cells become senescent, eventually dying by apoptosis leading to them being shed into the lumen. These colonic epithelial cells are clonal, arising from a stem cell population at the base of the crypts, and divide and differentiate as they migrate up the crypt. This process is carefully regulated to achieve a fine balance between a stem cell entering into the poliferative stage, at the bottom of the crypt, and cells dying by apoptosis, at the top (Lipkin et al., 1963; Loeffler et al., 1986). Such a homeostatic process is vital to maintain a healthy colonic epithelium. Any deregulation of the expansion of cell numbers in the proliferative zone could lead to local excesses of growth and development of tumours as proposed in the model of cancer progression by Tomlinson and Bodmer, who used colorectal cancer as an example (Tomlinson and Bodmer, 1995).

1.2.4 Progression to Colorectal Cancer.

Colorectal neoplasia occurs as a result of the pathological transformation of normal colon epithelium into benign adenomatous polyps which may then eventually lead to full invasive cancer. An early supporter of this idea was Morson who describe a biological progression, taking between 15 and 20 years, through a series of distinct morphological and histological stages (Morson, 1962). This progression has been termed the 'adenoma-carcinoma sequence' (Muto et al., 1975). Histologically, the development of microscopic areas of irregular glandular architecture, with no evidence of disordered growth or dysplasia

(termed 'aberrant crypt foci') have been suggested as areas likely to develop benign neoplasic polyps that may precede the outgrowth of a tumour (Nucci et al., 1997).

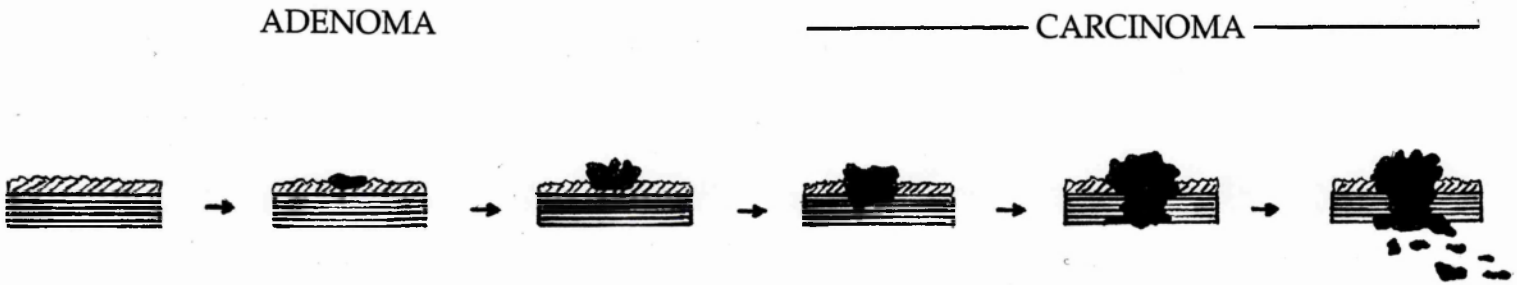
The earliest clinically identifiable event is the presence of polyps. These are tissue protrusions of the mucosa layer that extend into the lumen above the mucosal surface and arise from abnormal mucosal maturation and inflammation and are also seen at sites of regeneration following injury. They have been termed 'adenomatous polyps' or 'adenomas' (Norris, 1983).

In adenomas there appears to be continual cell proliferation, particularly in the proliferative zone of the crypts, which can result in this zone extending upwards into the upper maturation zone, with the proliferating epithelial cells showing delayed maturation. In addition, this continual disordered proliferation can lead to downward infolding of the crypt wall, causing a breakdown of the normal crypt architecture, sometimes characterized as a branching pattern in an individual crypt which can lead to the appearance of apparent collisions of adjacent crypts (Novelli et al., 1996). Adenomas in the colon have been classified according to the histological appearance of the polyp, with regard to the combination of tubular glands and villous projections, and the degree of dysplasia. The three types are: tubular, tubulovillus and villous adenomas. Those adenomas thought to have the highest malignant potential exhibit a high degree of dysplasia with a villous histology, and a size of greater than 2 cm may be an indicator (Muto et al., 1975).

The first indication of malignancy is the invasion through the basement membrane into the lamina propria, followed by further penetration into the muscularis mucosal layer. This layer has good connections with the lymphatic system into which the developing tumour can spread leading to regional lymph node metastases. Continued growth of the tumour may eventually invade the submucosal layer and enter the blood system leading to metastases developing in the liver, lungs and bones, as illustrated in Figure 3A.

FIGURE 3A

A SCHEME FOR THE ADENOMA TO CARCINOMA PROGRESSION, INDICATING AT WHICH DUKE'S STAGE MUTATIONS OCCUR IN APC, K-RAS, P53, hMLH1 and hMSH2



Pathologists have classified colorectal tumours according to the architectural and structural appearance of the tumour. The most widely used classification, however, is based more on the observation that colorectal tumours first spread circumferentially gradually invading the underlying tissue layers, with paracolic lymph node involvement being an early event, and haematogenous spread to other organs, a much later event. This is the Dukes staging classification (Dukes and Bussey, 1958) (see Table 1 and Figure 3A).

TABLE 1
DUKES STAGING CLASSIFICATION.

Dukes A	Lesions confined to mucosa/submucosa and no invasion of the muscularis propria.
Dukes B1	Lesions penetrate into the muscularis propria.
Dukes B2	Lesions penetrate through the muscularis propria
Dukes C1	Lesions have lymph node metastases, but the tumour is confined to the bowel wall.
Dukes C2	Lesions have lymph node metastases, but the tumour extends through the bowel wall.
Dukes D	Lesions have metastatic spread to distant organs.

The prognosis of these various stages range from >95% survival for 5 years for Dukes stage A patients to only a 10% survival for 5 years for Dukes stage C2 patients. [Stage B patients have about 80% survival for 5 years and stage C1 approximately 40% survival for the 5 years (Dukes and Bussey, 1958)].

1.2.5 Molecular Genetics of Colorectal Cancer.

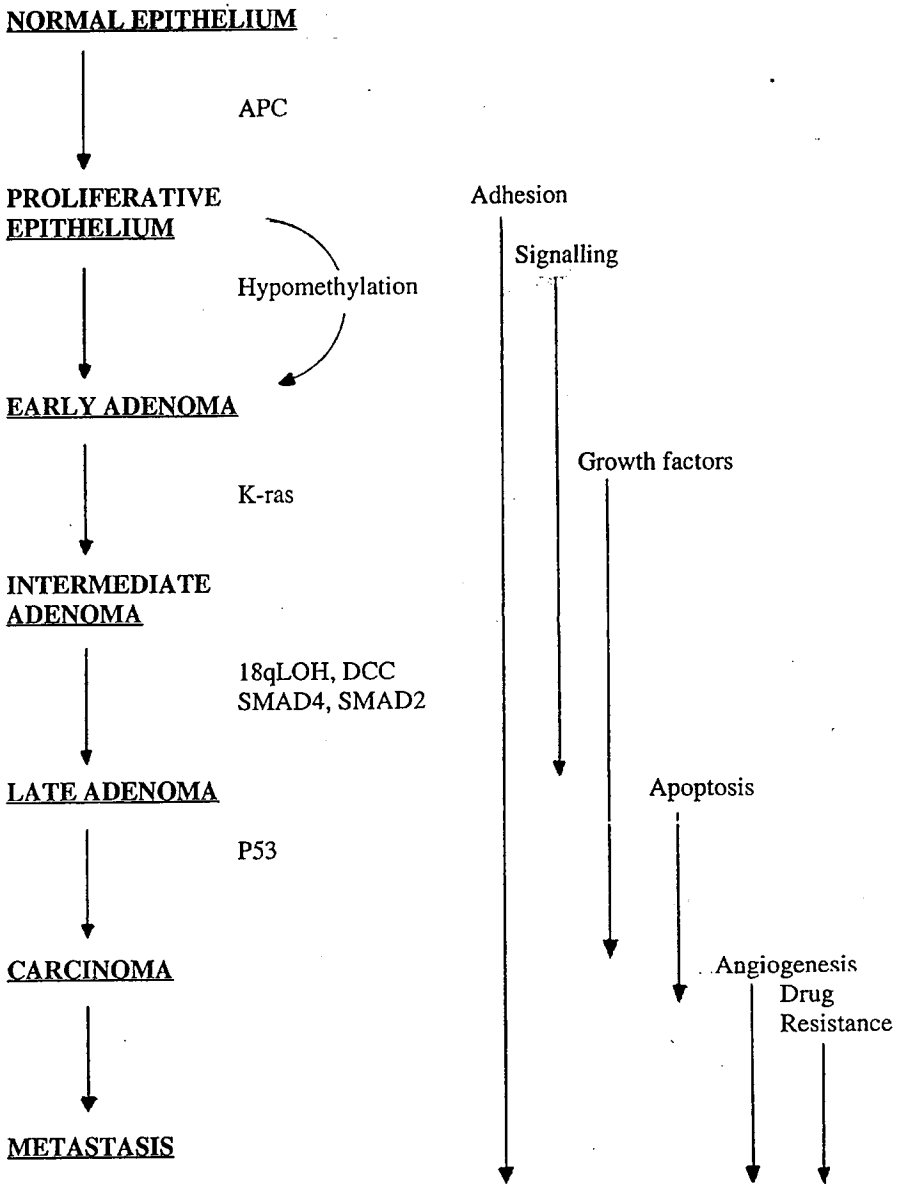
Colorectal cancer is a genetic disease at the cellular level and like all other cancers is characterized by genetic mutation followed by selection. It is not a single event, but a multistep process, that may take many years to develop. The various stages of the evolution of colorectal cancer that have been described and

classified by morphology and histology, can be largely explained by particular genetic changes. These can take the form either of mutational events, which alter and delete/insert nucleotides in the DNA sequence, or epigenetic events, such as methylation changes, that although do not change the genetic sequence, have a profound effect on gene expression. Whatever the molecular change is, they impart a proliferative advantage to the cell and contribute to the development of a malignant phenotype in the form of excessive cell growth, interruption of programmed cell death and local invasion as well as the ability to form distant metastases.

Progression to a cancer involves a stepwise accumulation of different mutational events, with each step conferring a selective advantage to the cell. An initial mutational event in a single cell, acquired somatically or inherited, may confer a selective growth advantage to that cell, but at this early stage may not outgrow its neighbours. Later (possibly after many years) further mutational events occur which can confer a selective advantage to the cell, but may not significantly increase cell numbers. Tomlinson and Bodmer, using the proliferating cells of the colonic crypt as an example, suggested that only after the accumulation of four or more such mutational events, will exponential growth occur leading to the establishment of a tumour (Bodmer, 1999; Tomlinson and Bodmer, 1995). The malignant phenotype appears to result from the mutational activation of oncogenes and the mutational inactivation of tumour suppressor genes and to involve at least four to five genes. These mutations can occur in an ordered sequence but it is the accumulation of these mutations that leads to tumour development, not necessarily the order of events (Bodmer, 1999; Fearon and Vogelstein, 1990). Figure 3B indicates some of the genes involved in the development of colorectal cancer and the stage at which they are thought to occur (Fearnhead et al., 2001; Fearon and Vogelstein, 1990; Woodford-Richens et al., 2001).

FIGURE 3B

The adenoma to carcinoma progression (showing key functions selected for at different stages of tumour progression).



1.2.6 Loss of HLA Expression on Colorectal Tumours.

The first report of a human tumour cell lacking expression of HLA class I described failure to express mature HLA-A, -B and -C antigens on the cell surface of the Burkitts lymphoma cell line, Daudi (Nilsson et al., 1974). Further work indicated this complete loss of HLA class I expression was due to the failure of Daudi cells to synthesize β_2-microglobulin. This lack of expression, on a cell type that would normally express HLA class I, may have been selected for during tumour development with the advantage of resistance to T cell immune attack (Arce-Gomez et al., 1978). Later the colorectal cell line, LoVo, was also shown to lack HLA class I expression and to have a similar loss of β_2-microglobulin synthesis (Brodsky et al., 1979b; Travers et al., 1982), though at this time the cause of the loss of β_2-microglobulin expression remained unknown. In addition, HLA class I was reported not to be detectable on choriocarcinomas, which are derived from progenitor cells, the trophoblasts, that are known to lack HLA class I expression. These cells have coodinately switched off HLA-A -B and -C expression, while still synthesizing and secreting β_2-microglobulin (Travers et al., 1982).

The generation of monoclonal antibodies, at first with specificities for β_2-microglobulin and monomorphic determinants of HLA class I (Brodsky et al., 1979b) and later with polymorphic reactivity with individual HLA class I alleles (Fauchet et al., 1984), allowed a more detailed study of HLA class I tissue distribution. Immunohistochemical analysis of tissue sections revealed HLA class I expression to be lost in examples of quite different tumours. For example, 6% of hepatocellular carcinomas (Paterson et al., 1988), 19% of ovarian tumours (Kabawat et al., 1983), 20% of non-Hodgkins Lymphomas (Moller et al., 1987) and 8% of colorectal carcinomas (Richman, 1987) showed complete loss of HLA class I expression. In a small study of 9 colorectal cell lines Makin (Makin, 1985) was unable to detect HLA class I in 5 of the cell lines when screening them by ELISA, using antibodies W6/32 (Barnstable et al., 1978) and BBM.1 (Brodsky et al., 1979a) which recognize mature HLA-A, -B and -C complexed with β_2-microglobulin and β_2-microglobulin respectively. A more detailed analysis of

HLA class I allele expression on colorectal tumours, using a panel of monoclonal antibodies, showed 1 of the 30 tumours studied lacked all HLA class I expression (Smith et al., 1989). This was determined by the lack of reactivity of the tumour cells with antibodies W6/32 and BBM.1, however, this tumour showed the presence of free HLA-A, -B and -C heavy chains in the cytoplasm by positive reactivity with antibody HC10 (Stam et al., 1986). Three other tumours in this study showed down-regulation of HLA class I expression by very weak or focal staining with the antibodies. However, in the same study frequent loss of individual HLA heavy chains was seen. Three out of 13 tumours had specifically lost HLA-A2 expression and another 4 of the13 had lost HLA-A2 and HLA-Bw4, probably indicating a haplotype loss in these 4 tumours.

In a similar study of 30 colorectal carcinomas, loss of reactivity with W6/32 was seen in 30% of tumours, but this loss of reactivity was not always associated with a loss of expression of β_2-microglobulin (Rees et al., 1988). Using tumour and corresponding normal epithelium from each patient, 15 of these individuals were shown to type as HLA-A2, but 4/15 of the tumours showed complete loss of HLA-A2 expression.

In a larger study of 70 consecutively collected colorectal tumours, 9 (12%) showed complete loss of surface expression of HLA class I (judged by lack of reactivity with W6/32) and an additional 2 (4%) of tumours had partial loss, demonstrated by focal pockets of tumour cells lacking reactivity with W6/32 (Kaklamanis et al., 1992). Detailed analysis with a panel of monoclonal antibodies revealed 5/9 of the tumours showed neither reactivity at the cell surface nor cytoplasmically with any of the reagents recognizing HLA class I and β_2-microglobulin. Two tumours in this study expressed cytoplasmic HLA-A heavy chain, but lacked HLA-B and -C and β_2-microglobulin, and one case lacked all HLA class I heavy chains, but expressed cytoplasmic β_2-microglobulin. In addition, one tumour expressed free HLA class I heavy chains in the cytoplasm, but no β_2-microglobulin was present. These results suggest that the loss of surface expression of HLA antigens can be the result of different genetic mechanisms affecting one or more of the HLA alleles causing either the loss of

expression of one particular allele product or loss of a complete haplotype of HLA-A, -B and -C, which for HLA would represent LOH. The loss of β_2-microglobulin expression would, however, require two separate events to occur presumably, though not necessarily, at different times (Smith, 1990).

Several attempts have been made to address the question of what sort of genetic event leads to the loss of expression of HLA antigens. When searching for the β_2-microglobulin gene, Goodfellow *et al* localized it to chromosome 15. This study included the lymphoblastoid cell line Daudi and it was noted that in Daudi/mouse hybrids there was a lack of expression of β_2-microglobulin. They concluded that this was due to a genetic change in the region encoding β_2-microglobulin (Goodfellow et al., 1975a). Lopez-Novet *et al.* showed loss of expression of HLA-A, -B and -C/β_2-microglobulin in 14% of colorectal tumours, by the lack of reactivity with W6/32 (Lopez-Nevot et al., 1989). They reported heterogeneous expression in the positive tumours, but found no correlation between HLA class I expression and tumour grade. DNA from tumours and, for control purposes, from surrounding normal mucosa, were digested with the isoschizomic enzymes *Msp*I and *Hpa*II, which are differentially sensitive to methylation of CCGG sequences. The resulting RFLP's were analyzed by Southern Blotting using an HLA-class I specific probe. No differences were reported between tumour and paired normal DNA indicating that there was no deletion of genes and no differences in methylation at the restriction sites used.

In a detailed study of 15 colorectal carcinomas which had lost HLA class I/β_2-microglobulin expression, Momburg and Koch investigated the levels of mRNA for HLA class I and β_2-microglobulin (Momburg and Koch, 1989). Using specific probes for HLA-B8 and β_2-microglobulin, in *in situ* hybridization studies, they demonstrated that in three tumour sections no message for β_2-microglobulin was present, compared to surrounding normal mucosal cells, whilst detectable amounts of message for HLA class I was present in both cell types. However, these studies were not able to explain the absence of the β_2-microglobulin mRNA which was suggested could be either due to down regulation or instability of the

transcripts. These studies were in contrast to the loss of β_2-microglobulin expression in the Daudi cell line, where mRNA has been detected, and the failure to express explained by the presence of a mutation in the initiation, ATG, codon of the β_2-microglobulin gene sequence (Ploegh et al., 1979; Rosa et al., 1983).

When typing a panel of 30 colorectal cell lines, Browning *et al.* reported a higher than expected number of cell lines that appeared to be homozygous for HLA-A (Browning et al., 1993). A subsequent study, which included data on HLA-B and –DR, suggested that up to 15% of these cell lines had an entire haplotype loss in the HLA region (Browning et al., 1996). The colorectal cell line PC/JW, and a corresponding normal B cell line (JWC) were included in this study. A complete HLA haplotype loss was demonstrated when comparing PC/JW with the normal JWC. Another of the colorectal lines, LS411, showed only a single allele loss for HLA-A11 when compared to RN, a corresponding normal B cell line. Conversely, LS174T and HCA-7 typed as HLA-A2, -A30 and HLA-A1, -A2 respectively, but LS174T was shown to lack expression of -A2 and HCA-7 lacked expression of -A1, when each line was analyzed with allele specific antibodies. In both cases the relevant mRNA's appeared to be detected by RT-PCR.

Attempts to re-express HLA class I have been reported with the cell line DLD-1 which lacks β_2-microglobulin expression. Browning *et al* demonstrated re-expression of HLA, as indicated by reactivity with the antibody W6/32, after infection of DLD-1 with a recombinant vaccinia virus encoding the human β_2-microglobulin gene (Browning et al., 1996). Toshitani *et al* established two stable transfectants of DLD-1, one expressing a (β_2-microglobulin/HLA-A2) construct and a second, transfected with a plasmid that contained only β_2-microglobulin, was shown to re-express endogenous HLA-A2 associated with the transfected β_2-microglobulin. This was determined by reactivity with antibodies W6/32 (anti HLA-A, -B, & -C/β_2-microglobulin) and BB7.2 (anti HLA-A2). In addition, this re-expression of endogenous HLA-A2 was upregulated after treatment with γIFN (Toshitani et al., 1996).

1.3 Research Aims.

At the time this thesis was started (1994), evidence for the loss of expression of HLA class I antigens on tumour cells was based on immunohistochemical studies. Most of these used the monoclonal antibodies W6/32 and BBM.1 that recognize surface expression of HLA-A, -B and -C complexed with β_2-microglobulin and β_2-microglobulin, respectively. In addition, evidence for losses of single HLA allele products had been established with allele specific antibodies, several of which had become available. In addition, the lymphoblastoid cell line Daudi, was the only reported case where a specific genetic event had been identified that lead to the complete loss of β_2-microglobulin synthesis, and hence lack of HLA class I expression at the cell surface.

In addition, several model systems, using gene constructs, have been reported for the study of the re-expression and peptide loading of HLA class I molecules in cells lines lacking HLA class I expression. These models have established stable transfectants using plasmid vectors, that allow continuous expression of HLA class I or HLA class I/β_2-microglobulin constructs. With the advent of inducible expression systems, we sort to establish a cell line with an inducible expression of an HLA-A*0201 /β_2-microglobulin construct. Such a system would allow the analysis of re-expression, peptide loading and functional status of an HLA class I/β_2-microglobulin construct in cells induced to express the construct, with the useful availability of un-induced cells to act as negative controls in studies of peptide presentation.

The research aims of this thesis were:

1) To investigate, in a panel of established colorectal cell lines, precise mutational events in the β_2-microglobulin gene that could lead to the loss of expression of all HLA class I.

2) To extend the investigation, for mutations in the β_2-microglobulin gene, to include a large collection of tumour cells derived from different carcinomas and to include where possible control data from paired normal tissue.

3) To investigate if the occurrence of mutations in β_2-microglobulin genes correlated with any other genetic event, such as microsatellite instability which is associated with errors in the DNA mismatch repair pathway.

4) To identify, at the genetic level, mutations in specific HLA class I alleles that could lead to the loss of expression of that allele product.

5) To establish a model inducible system for the expression of an (HLA-A*0201/β_2-microglobulin) construct in cell lines lacking expression of HLA class I. These experiments were initially attempted in the human colorectal cell line DLD-1, with additional work carried out in the CHO cell line.

6) To study parameters, such as concentration and time scale of stimulation by the inducer, that would influence the inducibility of the (HLA-A*0201/β_2-microglobulin) construct. In addition, to verify the functional status of the induced construct by the ability to present specific peptides to an HLA-A2 restricted, peptide specific, cytotoxic T cell line and demonstrate cell lysis in a cytotoxic T cell killing assay.

CHAPTER 2

MATERIALS AND METHODS

2.1 CELL LINES.

2.1.1 Cell Line Culture Techniques.

Approximately $5x10^6$ cells were cultured in 10ml of either Dulbecco's modified E4 medium (DMEM) supplemented with 10% heat inactivated (55°C for 30 minutes) Foetal Calf Serum (FCS) or Iscove's modified Dulbeccos medium (Gibco/BRL, UK) supplemented with 10% heat inactivated FCS. Antibiotics, Penicillin 200 IU/ml and Streptomycin 200 µg/ml, were added to both media types used. Usually, 75 sq.cm flasks were used, incubated in a humidified incubator at 37°C with 10%CO_2.

When adherent cells were approaching a confluent culture they were split into fresh cultures using one of two different methods:
Method 1) The culture medium was removed and the monolayer washed with 10ml of Phosphate buffered saline (PBSA), then washed a second time with Ca deficient medium (DMEM modified for suspension cultures, Flow/ICN, UK). The culture was then set up with 4ml per 75 sq.cm flask of Ca deficient medium supplemented with antibiotics and cultured overnight at 37°C in 10%CO_2. By next day most of the cells appeared rounded and some had detached. To increase cell detachment, the flasks were tapped gently and any remaining attached cells detached using a rubber policeman. The resulting cell suspension was then set up in a new culture flask containing one of the culture media mentioned above.
Method 2) The culture medium was removed and the cells washed with 10ml of PBSA. Then 4ml of pre-warmed (37°C) Trypsin/EDTA solution was added and the culture incubated for several minutes at 37°C. As soon as the cells were

detached, 10ml of culture medium containing 10% FCS was added and the cell suspension transferred to a 15ml centrifuge tube. The cells were then recovered by centrifuging at 1500rpm for 5 minutes at room temperature. The supernatant was removed and the cells then set up in a fresh culture flask containing the appropriate culture medium. Cells were normally split in the range of 1:4 to 1: 20 during passage.

Lymphocyte and other cell lines which grew in suspension were normally cultured in Roswell Park Memorial Institute (RPMI) medium No 1640 supplemented with 10% heat inactivated FCS, Penicillin 200 IU/ml and Streptomycin 200 μg/ml. Cultures were incubated in a humidified incubator at 37°C with 5% CO_2. When these cultures reached confluency, an aliquot of the cells was removed and added to a new flask containing fresh medium, normally splitting in the range of 1:10 to 1: 50 depending on the cell line.

2.1.2 Colorectal Cell Lines used in these studies.

The colorectal cell lines used in these studies were as follows:

C10	Established in the Cancer & Immunogenetics Lab, CRuk, Oxford
C32	Established in the Cancer & Immunogenetics Lab, CRuk, Oxford
C70	Established in the Cancer & Immunogenetics Lab, CRuk, Oxford
C75	Established in the Cancer & Immunogenetics Lab, CRuk, Oxford
C80	Established in the Cancer & Immunogenetics Lab, CRuk, Oxford
C84	Established in the Cancer & Immunogenetics Lab, CRuk, Oxford
C99	Established in the Cancer & Immunogenetics Lab, CRuk, Oxford
C106	Established in the Cancer & Immunogenetics Lab, CRuk, Oxford
C125PM	Established in the Cancer & Immunogenetics Lab, CRuk, Oxford
CaCo2	(Fogh et al., 1977)
CC07	(Katagiri, 1987)
CC20	(Greenhalgh and Kinsella, 1985)
Colo201	(Semple et al., 1978)
Colo205	(Semple et al., 1978)
Colo206	(Semple et al., 1978)

Colo320DM	(Quinn et al., 1979)
Colo678	Not Published From DSMZ-Deutsche, Deposited by Moore, E.G., USA
Colo741	ECACC Collection No 93052621
CX-1	Not Published From DSMZ-Deutsche, Deposited by DKFZ Tumorbank
DLD-1	(Tibbetts et al., 1977)
GP2d	(Solic et al., 1995)
GP5d	(Solic et al., 1995)
HCA-7	(Kirkland, 1985)
HCA-46	(Kirkland and Bailey, 1986)
HCT-15	(Tibbetts et al., 1977)
HCT-116	(Brattain et al., 1981)
HRA-19	(Kirkland and Bailey, 1986)
HT-29	(Fogh and Trempe, 1975)
HT-55	(Goldin and al, 1981)
LIM1863	(Whitehead et al., 1987)
LoVo	(Drewinko et al., 1976)
LS-174T	(Tom et al., 1976)
LS-180	(Tom et al., 1976)
LS-411	(Suardet et al., 1992)
LS-1034	(Suardet et al., 1992)
PC/JW	(Paraskeva et al., 1984)
SKCO-1	(Fogh and Trempe, 1975)
SW48	(Leibovitz et al., 1976)
SW403	(Leibovitz et al., 1976)
SW480	(Leibovitz et al., 1976)
SW620	(Leibovitz et al., 1976)
SW837	(Leibovitz et al., 1976)
SW948	(Leibovitz et al., 1976)
SW1116	(Leibovitz et al., 1976)
SW1417	(Leibovitz et al., 1976)
SW1222	(Leibovitz et al., 1976)
T-84	(Murakami and Masui, 1980)
VACO-4A	(McBain et al., 1984)
VACO-4S	(McBain et al., 1984)
VACO-5	(McBain et al., 1984)

VACO-10MS (McBain et al., 1984)
WIDR (Fogh and Trempe, 1975)

2.1.3 Other (non-colorectal) cell lines used in these studies.

Non-colorectal cell lines used include the following lines

Lymphoblastoid
EVA 1224 (Kirkland, 1985)
BRISTOL-8 From ECACC. Cell line No 85011436

Miscellaneous
CHO.K1 From ATCC. Cell line ref No CCL61

2.2 TUMOUR SAMPLES.

Colorectal tumour samples were obtained with the co-operation of Mr Neil Mortensen and Mr Kettlewell (Department of Surgery, John Radcliffe Hospital, Oxford) and the material was identified and dissected by Dr Lucas Kaklamanis (Department of Pathology, John Radcliffe Hospital, Oxford). Same additional colorectal tumour sample were provided by Dr Ian Frayling (St Marks Hospital, London).

Ovarian tumours were obtained from Dr Inez Cooke, Ovarian cancer Group, ICRF, Institute of Molecular Medicine, Oxford.

Samples from Breast tumours were kindly supplied by Prof. A. Harris, ICRF Molecular Oncology Laboratory, Weatherall Institute of Molecular Medicine, Oxford.

Samples of DNA from Lymphoma patients were obtained from Prof. D Mason, Haematology Laboratory, John Radcliffe Hospital, Oxford

Melanoma samples were obtained from Dr Catriona MacGeoch, Human Genetic Resources Laboratory, ICRF Clare Hall Laboratories, South Mimms, Hertfordshire and Prof. Ian Hart, Richard Dimbleby ICRF Department of Cancer Research, St. Thomas' Hospital, London.

2.3 IMMUNO-ASSAYS.

2.3.1 Immunofluorescence and Flow Cytometry.

Cell surface membrane expression of various antigens was examined using immunofluorescence followed by analysis on a Flow cytometer. Briefly, adherent cell cultures were trypsinized and a washed cell pellet (10^6 cells) produced as described above. Non-adherent cells were washed twice with DMEM before assaying. 50µl of antibody dilution (10µg/ml) was then added to the cells and incubated on ice for 30 minutes. A negative control containing no antibody was also prepared. After washing the cells twice in cold DMEM/2% heat inactivated FCS, a 1:20 dilution of FITC conjugated goat-anti-mouse (50µl) was added followed by incubation on ice, in the dark, for 30 minutes. Cells were then washed twice in cold DMEM/2% heat inactivated FCS and finally resuspended in 500µl of 1% paraformaldehyde in PBS. The cells were stored at 4°C in the dark until analysis on a Flow cytometer. Cells were analyzed using a FACScan (Beckton Dickinson) for one colour fluorescence (FL1) with side scatter (SSC). Antibodies used in these studies were:

BBM.1 (β_2-microglobulin) (Brodsky et al., 1979a)

BB7.2 (HLA-A2/AW69) (Brodsky et al., 1979b)

HCA2 (HLA Heavy chain, A locus) (Stam et al., 1990)

HC10 (HLA Heavy chain, B & C loci) (Stam et al., 1986)

L368 (β_2-microglobulin) (Lampson et al., 1983)

W6/32 (Mature HLA-A, B & C couple with β_2-microglobulin) (Barnstable et al., 1978)

Rabbit anti-mouse Polyclonal (Dako, Denmark)

Goat anti-mouse Polyclonal/FITC (Dako, Denmark)

2.3.2 Enzyme-Linked ImmunoSorbant Assay (ELISA).

The GAG ELISA assay used in these studies was based on that described earlier (Durbin and Bodmer, 1987). Adherent cells were grown up on microtiter plates (Becton Dickinson) to within 3-5x10^4 cells per well. The cells were then fixed by incubation with 0.025% glutaraldehyde (Sigma) in PBSA for 30 minutes at room temperature. Background blocking was then achieved by incubation with 0.1% gelatin in PBSA for at least 1 hour at room temperature or overnight at 4°C. For the assay, plates were washed x3 with PBSA/0.2% casein then 50µl of antibody dilution (10µg/ml) added per well and incubated at room temperature for 1 hour. Plates were then washed x3 with PBSA/0.2%casein/0.2% Tween 20 (Sigma). 50µl of 1:100 dilution of Rabbit-anti-mouse (Dako, Denmark) in DMEM/10% FCS was then added per well and incubated for 1 hour at room temperature. Plates were washed x3 with PBSA/Casein/Tween 20, followed by the addition of a 1:750 dilution of GAG complex (50µl) per well and incubated as above. The GAG complex consisted of antibody 4C7, which specifically reacts with β-galactosidase, coupled with β-galactosidase, Sigma. Plates were washed three times as described and finally 100µl of substrate solution (4-methlyumbelliferyl-β-D-galactoside dissolved in 1mM Mg Cl_2/100mM β-mercaptoethanol) was added per well and incubated for 30 minutes at room temperature in the dark. Plates were read in a Microfluor plate reader (Dynatech, UK).

2.3.3 Cytotoxic T Lymphcyte Assay (CTL Assay).

A human HLA-A2 restricted, influenza A matrix specific, CTL line was generated from blood donor PG (Gotch et al., 1987). CTL activity was measured in a standard ^{51}Cr release assay. Target cells were cultured either with Ponasterone A (1μM) for 24 hours to induce HLA expression or with fresh culture medium only, then trypsinized and washed in culture medium. These cells were then labeled with 100μCi of ^{51}Cr (Amersham, UK) for 1 hr at 37°C, and washed twice with culture medium. These cells were then incubated for 1 hr with flu matrix peptide (GILGFVFTL, 1μM) and washed x3 with culture medium. For the assay, 5 x 10^3 target cells per well were incubated for 5 hrs with either CTL's, known to be against the peptide target, at various Effector:Target (E:T) ratios, medium alone (spontaneous release) or 5% Triton X-100 (maximum release). Aliquots (20μl) of supernatant were harvested and ^{51}Cr release determined using a γ-plate counter (LKB, UK). The percentage of specific lysis was calculated as [(release by CTL's) – (spontaneous release)] x 100/[(maximum release) – (spontaneous release)].

2.3.4 Immunocytochemistry.

Immunocytochemical detection was performed using the alkaline phosphatase method as described by Cordell *et al.* (Cordell et al., 1984) Briefly, frozen tissue sections (7μm thick) were fixed in cold acetone for 10 minutes at room temperature and air dried overnight. Primary mouse monoclonal antibody (50μl) was added to the dry sections in a humidified chamber for 30 minutes followed by three washes with Tris buffered saline (TBS) pH 7.4. Anti-mouse immunoglobulin G (DAKO) and APAAP complex (DAKO), both diluted 1:50 in TBS, were added sequentially and each incubated for 30 minutes at room temperature. Each step was separated by a washing period of 5 minutes with TBS. These steps were repeated to enhance the intensity of the final staining.

After a final wash with TBS the alkaline phosphate substrate was added and incubated for 15 minutes at room temperature. Sections were counterstained with haematoxylin and mounted in an aqueous mounting medium.

2.4 MOLECULAR BIOLOGICAL METHODS.

2.4.1 Extraction of DNA and mRNA.

DNA extraction from cell lines was performed by Nucleon II DNA extraction kit (Tepnel, UK) according to the manufacturers instructions. Briefly, this involved lysing the cells in a buffer containing SDS followed by protein precipitation with sodium perchlorate, followed by a chloroform separation. Any remaining protein was removed by the addition of a silica slurry, which bound the protein, followed by centrifugation. The isolated DNA was recovered by ethanol precipitation and washing with 70% ethanol and finally resuspended in sterile H_2O. DNA concentrations and purity were estimated by measuring appropriate dilutions in a spectrophotometer at 260nm and 280nm. Some early preparations of cell line DNA were extracted by using an Applied Biosystems model 340A nucleic acid extractor.

DNA from fresh tumours and normal colon epithelial cells was extracted from biopsies obtained at the time of surgery. On arrival in the laboratory samples were divided into two portions, one portion used for establishment of a cell line and the second snap-frozen in liquid nitrogen and cryopreserved until required. DNA extraction was performed as described above using the Nucleon II system. A slight modification was used during the cell lysis step. Tumour samples were cut into pieces as small as possible (approximately 0.5mm) using two scalpels and placed into a tube with 6ml of lysis buffer that contained twice the usual concentration of SDS. Cell lysis was performed by rotating the preparation for 17 hours at room temperature, then sodium perchlorate was added and the extraction continued as described above.

mRNA was extracted from tissue and cell lines using a Messenger RNA Isolation Kit (Stratagene, California, USA). The basic protocol was to lyse the cells in 5ml of a proprietary buffer containing Guanidine thiocyanate and β-mercaptoethanol at room temperature. The DNA in this solution was then sheared by passing the lysate through a 21-gauge needle. 10 ml of a proprietary elution buffer was then added and the whole sample centrifuged at 12,000g to remove cell debris. The supernatant was mixed with a slurry of Oligo (dT) cellulose resin and incubated for 15 minutes, at room temperature, on a rotary stirrer, and transferred to a syringe fitted with a glass fibre filter at the bottom. The slurry was washed three times with a high salt buffer followed by one wash with low salt buffer. Finally the polyA RNA was eluted from the resin with 400µl of warm (68°C) elution buffer. The mRNA was then precipitated by adding 1/10 volume of sodium acetate (3M) and 2.2 volumes of cold ethanol and recovered by centrifugation (13,000rpm in an Eppendorf mini centrifuge). The mRNA was washed once with 70% ethanol and finally resuspended in RNase free water. Samples were stored at -80 °C until use.

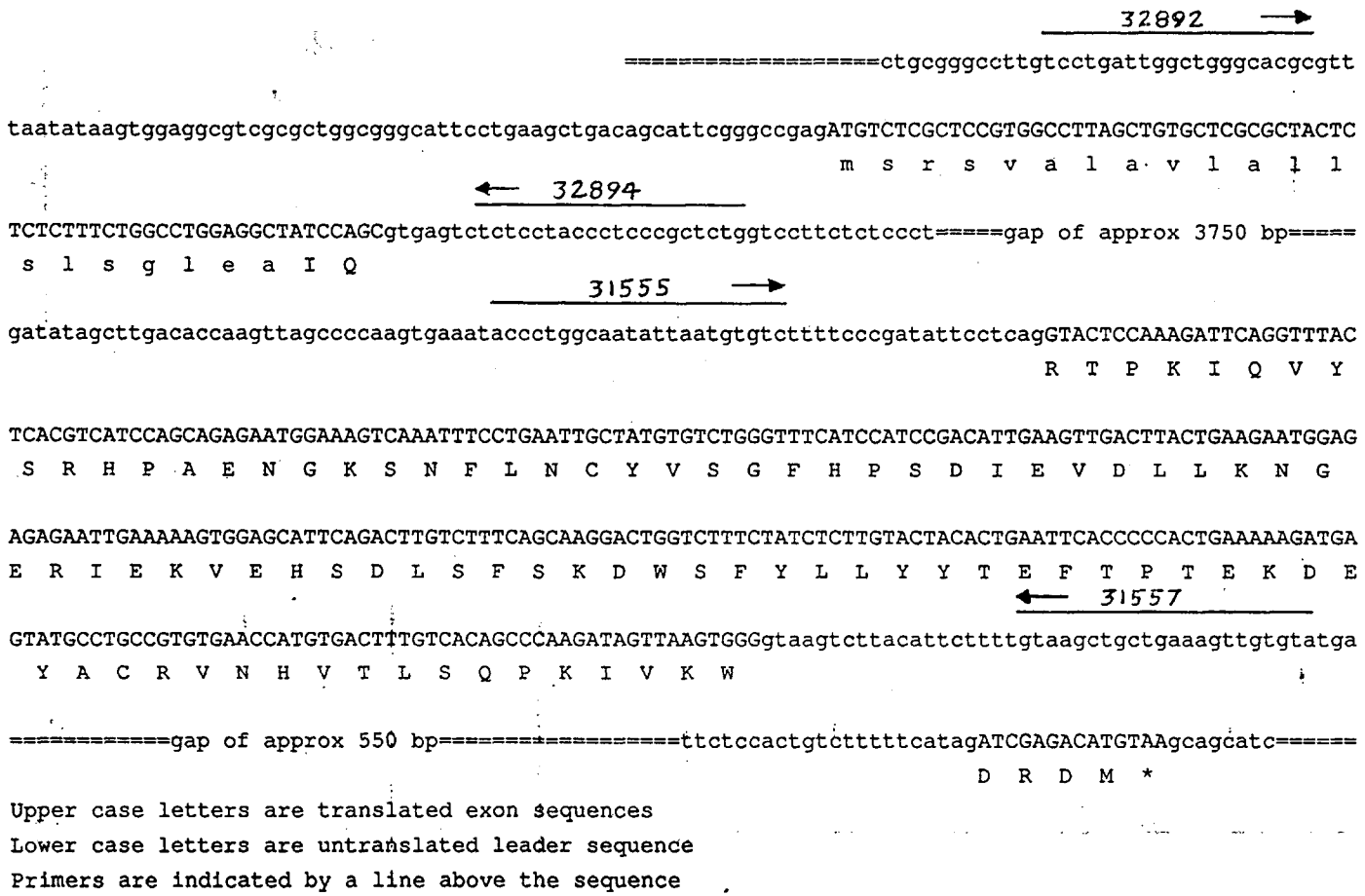
2.4.2 Polymerase Chain Reaction (PCR).

A) β_2-Microglobulin

PCR for β_2-microglobulin analysis was performed in 50µl reactions containing all four NTP's (each at 200µM), 0.2µM of the appropriate primer combination, as shown in Table 2, 1µg of genomic DNA, 1 unit of Amplitaq (Perkin-Elmer/Cetus) and either buffer A or B (primers 32892 and 32894 with buffer A for leader peptide/exon 1 and primers 31555 and 31557 with buffer B for exon 2). Buffer A contained 50mM KCl, 10mM Tris-HCl pH8.3, $MgCl_2$1.5mM and 0.25% Nonidet P40. Buffer B contained 10mM Tris-HCl pH8.3, 50mM KCl, 3mM $MgCl_2$ and 0.01% Triton X100. The mixes were over layered with one drop of light mineral oil (Sigma). Samples were amplified for 30 cycles in a thermal cycler (Hybaid, Teddington, U.K.) as follows; for primer combination 32892/32894; 92°C (30 secs), 68°C (30 secs) and 70°C (2 mins) and for primer combination 31555/31557; 92°C (30 secs), 60°C (30 secs) and 72°C (2 mins).

FIGURE 4

B_2MICROGLOBULIN SEQUENCE SHOWING THE LOCATIONS OF PRIMERS USED FOR PCR



Upper case letters are translated exon sequences
Lower case letters are untranslated leader sequence
Primers are indicated by a line above the sequence

TABLE 2

SEQUENCE OF PCR PRIMERS USED FOR β_2-MICROGLOBULIN ANALYSIS

Primer	Sequence (5'-3')
32892 Forward – Leader Pep/Exon 1	TCCTGATTGGCTGGGCACGC
32894 Reverse – Leader Pep/Exon 1	CAGAGCGGGAGGGTAGGAGA
31555 Forward – Exon 2	ACCCTGGCAATATTAATGTGTC
31557 Reverse – Exon 2	TACACAACTTTCAGCAGCTTAC

The genomic sequence of for β_2-micoglobulin is shown in Figure 4 with the location of primers used for β_2-micoglobulin analysis.

B) HLA-A*0101 and HLA-A*0101"null" alleles.

For analysis of the HLA-A*0101 and HLA-A*0101"null" alleles the same protocol and cycling conditions were followed as described for HLA class I typing published elsewhere (Tonks et al., 1999). The primers that distinguish between HLA-A*0101 and HLA-A*0101"null" have been published previously (Bunce et al., 1999), and those used in these studies are shown in Table 3.

TABLE 3

SEQUENCE OF PCR PRIMERS USED FOR ARMS-PCR ANALYSIS OF HLA-A*0101 AND HLA-A*0101"Null" ALLELES

Primer	Sequence (5'-3')
123381 Forward – HLA-A1 all alleles Intron 3 bp240-258	GACGGGAAGAGGATCCCTC
123385 Reverse - HLA-A*0101 Exon 4 bp 620-638	ATATGTGTCTTGGGGGGGT
123386 Reverse – HLA-A*0101"Null" Exon 4 bp 621-639	TATGTGTCTTGGGGGGGGT

C) Sequencing HLA-A alleles.

PCR conditions, using a pair of primers with HLA-A specificity, used to prepare templates for sequencing of the HLA-A*01 and HLA-A*02 alleles were as follows: 50µl reactions containing all four NTP's (each at 200µM), 0.2µM of each primer (Forward Primer 5'GACGGGAAGACGATCCCTC3' and reverse primer 5'CTGCAGGCCTGGTCTCCACG3'), 90ng of genomic DNA, 1 unit of Amplitaq Gold (Perkin-Elmer/Cetus) and 0.1 unit Pfu Taq (Stratagene) in a buffer containing 50mM KCl, 10mM Tris-HCl pH8.3 and $MgCl_2$1mM. The mixes were over-layered with one drop of light mineral oil (Sigma). Samples were amplified for 1 cycle of 95°C for 12 minutes followed by 30 cycles in a thermal cycler (Hybaid, Teddington, U.K.) of 92°C (30 secs), 68°C (30 secs) and 72°C (2 mins).

2.4.3 Agarose Gel Analysis.

Following PCR, a representative sample of the reaction was usually analyzed by gel electrophoresis. The gel consisted of 1-2% agarose (Gibco/BRL) in 1x TBE buffer (1 litre H_2O containing 10.8g Tris base, 5.4g Boric acid and 4ml 0.5M EDTA). Before the gel was cast, 3µl of ethidium bromide (10mg/ml) per 50ml gel was added to aid visualization of the PCR product under UV light. Samples (6µl) plus 1µl of loading buffer (0.2% bromophenol blue, 0.2% xylene cyanole and 50% glycerol in H_2O) were loaded onto the gel. Standard size marker was usually a *Pst*1 digest of *Lamda* DNA prepared in loading buffer. A running buffer of 1xTBE containing ethidium bromide, as above, was used and the gel runs at 100 volts at room temperature for approximately 30 minutes. PCR products were visualized under UV light on a transilluminator and recorded by *Polaroid* camera.

2.4.4 Single-Strand Conformation Polymorphism Analysis (SSCP).

Prior to SSCP analysis, PCR reactions were labeled with ^{32}P. An aliquot (1 µl) of PCR product was added to 25 µl of fresh PCR reaction mixture containing the

appropriate buffer and primer pairs. Additionally, 50μCi of [α-^{32}P]dCTP (specific activity, 3000Ci/mmol [1Ci=37GBq], Amersham, UK) were added. PCR amplification was then performed for 10 cycles, under the conditions described above for each primer combination.

For SSCP analysis, 1μl samples of the ^{32}P-labeled PCR products were added to 10μl of SSCP loading buffer containing 15mM EDTA, 0.05% SDS, and 50% (vol/vol) formamide. Samples were then denatured at 95°C for 5 minutes and super-cooled. Seven microlitres was loaded on to a 50cm-long non-denaturing SSCP gel and electrophoresed at 450volts at room temperature for 24 hours. Analysis of the leader peptide/exon 1 PCR product was achieved on an 8% polyacrylamide gel containing 17.5% (vol/vol) glycerol and 1xTBE buffer. For SSCP analysis of exon 2 PCR products, a 5% polyacrylamide gel containing 10% glycerol and 1xTBE buffer was used. The running buffer used in both cases was 1xTBE, and electrophoresis carried out at 400 volts for 20 hours at room temperature, after which the gels were dried and bands located by autoradiography.

2.4.5 Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR).

The analysis of mRNA from the cell lines EVA-1224 and HCA-7 were carried out using the Superscript™II (Reverse transcriptase-DNA polymerase combination) First Strand Synthesis System (Gibco/BRL, UK) followed by PCR using primers designed in the laboratory. For first strand synthesis, mRNA (approximately 2μg) was added to a solution of proprietary Random Hexamer Primers (3.5ng/μl) and heated to 70°C for 10 minutes, followed by supercooling on wet ice. The following were then added, 2μl of 10x Synthesis buffer (200mM Tris/HCl pH8.4, 500mM KCl) 1μl of 10mM dNTP's, 2μl 100mM DTT and 1μl Superscript™II (50units/μl) and incubated for 10 minutes at room temperature, 50 minutes at 42 °C and 15 minutes at 70 °C. To remove any remaining single strands the sample was incubated for 20 minutes at 37 °C with 40 units of RNaseH. This reaction was then diluted 1:4 with ddH_2O and used as a source of template in a 50μl PCR with the following conditions: all four NTP's (each at

200μM), 0.2μM of each primer (see below), 90ng of genomic DNA, 1 unit of Amplitaq (Perkin-Elmer/Cetus) all in a buffer of 16mM Ammonium Sulphate, 67mM Tris-HCl pH8.4, 6.7mM EDTA, 0.017% BSA and $MgCl_2$1mM. The mixes were over layered with one drop of light mineral oil (Sigma). Samples were amplified for 5 cycles of 95°C (50 secs), 70°C (50 secs), 72°C (50 secs), then 21 cycles of 95°C (50 secs), 63°C (50 secs), 72°C (50 secs), and a final 4 cycles of 95°C (50 secs), 55°C (50 secs), 72°C (50 secs), in a thermal cycler (Tetrad, MJR, USA). Primers used were:

AL-16 (5'GACCAGGAGACACGGAATA3')
AL-x (5'GCCCGTCCACGCACCG3')
AL-13 (5'TGGATAGAGCAGGAGGGT3')
AL-H (5'CAAGAGCGCAGGTCCTCT3')
61998 (5'GAGGGGCTTGGGCAGAC3')
46992 (5'CTTTACAAGCTGT3')
HLA3UTA (5'GAGGGAGCACAGGTCAGCGTGGGAAG3').

2.4.6 HLA Typing.

DNA was extracted from cell lines and fresh tumour samples as outlined in section 2.4.1. ARMS-PCR for HLA class I typing was performed as described elsewhere (Tonks et al., 1999) using a panel of 96 primer mixes designed to give a medium resolution of HLA-A, -B and -C alleles. Following PCR, amplicon detection was achieved using the Alkaline Mediated Differential Interaction (AMDI) method (Bartlett et al., 2001) were SYBR green 1 (Molecular Probes, UK) diluted 1:5,000 in 200mM 4-[cyclohexlamino]-1-butansulphonic acid (CABS, Sigma Aldrich, UK), pH12.7 was added to each reaction and PCR products detected by fluorimetry. Software developed in the laboratory by Mr Adam Lowe was used to interpret the data.

2.4.7 Microsatellite Analysis.

Microsatellite analysis was performed using four loci, on different chromosomes, containing dinucleotide repeat sequences. The loci used were D2S123 (chromosome 2), D10S209 and AFMb001 (chromosome 10) and D17S941 (chromosome 17). In some cases, microsatellite instability was confirmed by further analysis of the D13S175 (chromosome 13), D2S378 and D2S119 (chromosome 2) and D10S197 (chromosome 10). The primer sequences used were taken from the 1993-4 Genethon human genetic linkage map (Gyopay et al., 1994). The PCR reaction contained 50mM KCl, 10mM Tris/HCl(pH8.3), 1.5mM $MgCl_2$, 0.25% NP40, 200μM NTP's, 0.2μM of the appropriate primer combination, the 5' primer was labeled with a fluorescent tag, 1 unit Taq polymerase, and 0.5-1μg genomic DNA. Amplification was performed on a Thermocycler (Hybaid, UK) with 35 cycles of 90°C (1 min), 54°C (30 secs), 72°C (30 secs). PCR products were analyzed on an Applied Biosystems ABI337A DNA Genotyper. Samples with variability at two or more loci were classified as microsatellite unstable.

2.4.8 DNA Sequencing (Single Strand Method).

DNA sequencing of PCR products was performed using the direct solid phase sequencing system (Hultman et al., 1990). Briefly, a PCR product for β_2-microglobulin leader peptide/exon 1 was amplified as described in section 2.4.2, but with the 3' primer 32894 biotinylated. The whole of the PCR product was purified by running in a 2% low melting point agarose gel using TAE buffer (Sambrook et al., 1989) and the product visualized on a UV transilluminator. After removing the appropriate band, the DNA was recovered using a Mermaid kit (BIO 101 Inc., California, USA) according to the manufacturer's protocol. Single stranded DNA was then isolated using Streptavidin coated Dynabeads M280 (Dynal AS, Norway) as described (Hultman et al., 1990). Single stranded sequencing was then performed using a Sequenase II kit (USB Corporation, Ohio, USA) using 5' primer 32892 to prime the sequencing reaction. On completion of the reaction the samples were heated to 85°C for 5 min then super

cooled. The immobilized template DNA was then removed using a magnet (Dynal AS). Samples were electrophoresed in a standard 6% denaturing polyacrylamide gel, followed by gel drying and autoradiography. Sequencing in the opposite direction was achieved by using biotinylated 32892 primer in the PCR reaction and non-biotinylated 32894 to prime the sequencing reaction. β2-microglobulin exon 2 was sequenced in a similar way using the appropriate combination of biotinylated and non-biotinylated primers. However, in this case the DNA was recovered from agarose gel purification with a Geneclean II kit (BIO 101) because of the larger size (361 bp) of this exon product.

2.4.9 DNA Sequencing (PCR/Automated Method).

For sequencing by the automated ABI system, regions of DNA for sequencing were first expanded by PCR as described above in section 2.4.2. An aliquot of the PCR was analyzed by gel electrophoresis (as described in section 2.4.3) to establish that a clean single band had been produced. The product was purified using a Nucleon (Tepnel, UK) PCR 'Clean up' kit according to the manufacturers protocol and finally eluted in 5µl of water. The sequencing reaction was performed using the ABI Prism 'BigDye Terminator' Kit (Applied Biosystems, UK) with the following components in the reaction: template (from PCR reaction) 5µl, sequencing primer (50mg/ml) 1µl and BigDye reaction mix 4µl. This reaction was over-layed with one drop of mineral oil and then amplified in a thermal cycler (MJR, DNA Engine Tetrad) as follows: 25 cycles of 96°C (30 secs), 50 °C (15 secs), 60 °C (4 mins), then held at 15 °C. The extension product reaction was then ethanol precipitated and finally dissolved in 4µl of ABI sample buffer. Prior to analysis on an ABI 377 sequencer, the samples were denatured at 98 °C for 5 mins, then supercooled on iced water. Sequences were analyzed using the Sequencher 3.0 software (ABI PRISM).

2.4.10 Cloning and Sequencing.

In the case of sequencing directly from PCR products where two HLA-A loci were represented in the amplicon mix, clearer sequence data were obtained after cloning from this mix. PCR was performed as described above (2.4.2) and the reaction monitored for product purity by analyzing an aliquot on agarose gel electrophoresis (see 2.4.3). This PCR product was then cloned using the PCR-Script™ Amp Cloning Kit (Stratagene) following the manufacture's protocol. This procedure involves the blunt ending of the PCR product followed by ligation into the pPCR-Script Amp SK(+) vector. In this reaction a continuous supply of blunt ended cut vector is available from the activity of the restriction enzyme *Srf*1 which cuts at the 5'GCCC/GGGC3' site in the cloning region of the vector. The blunt ended PCR product is ligated into this site with the aid of T4 DNA ligase. The resultant plasmid is introduced into XL10-Gold Kan bacteria and plated out on to standard agar plates containing ampicillin, X-gal and IPTG. (The vector pPCR-Script Amp SK(+) is derived from pBluescript and contains the lacZ gene allowing blue white selection in the presence of X-gal and IPTG). After overnight incubation at 37 °C, white colonies were picked and individually grown up in 3ml cultures. Plasmids from these clones were isolated using the QIAprep miniprep kit system (Qiagen, UK) according to the manufactures instructions. The quality of the plasmid recovered from the miniprep extraction was sufficiently high enough to allow an aliquot to be included in the sequencing reaction without further purification. Usually the plasmid was sequenced using the ABI system described above in section 2.4.8.

2.4.11 Plasmid/Construct Development.

The (pcDNA-β_2M15-A2) construct containing full length β_2-microglobulin cDNA (including the leader peptide sequence), a $(GGGGS)_3$ linker and HLA-A2 cDNA has been described before (Toshitani et al., 1996). The restriction sites of construct (pcDNA-β_2M15-A2) were changed to a *NheI* site (5') using primer

5'CGGGCTAGCATGTCTCGCTCCGTGGCCTTA3' and a *Hind III* site (3') using primer 5'GCCCAAGCTTGAGGTCGACTCTAGAGGATCC3' in a PCR reaction using cloned *Pfu* DNA Polymerase (Stratagene, UK) in conditions recommended by the manufacturer. Cycling temperatures used were 30 cycles 95^0C (1 min), 65^0C (1min), 72^0C (2.5 mins) followed by 1 cycle of 72^0C (5 mins). Similarly, the restriction sites of a construct containing only the β_2Microglobulin gene (pcDNA-β_2M), reported by the same authors, were also changed by a similar PCR technique using the following primers: 5' primer 5'CGGGCTAGCATGTCTCGCTCCGTGGCCTTA3, containing a *NheI* site and 3' primer 5'GGGATCGAGACATGTAAAAGCTTTCA3' containing a *Hind III* site. Following PCR, each amplicon was digested with *Nhe I* and *Hind III*, then gel purified before they were ligated into the appropriately digested cloning site of vector pIND (Invitrogen, UK). These plasmids were designated (pIND-β_2M15-A2) and (pIND-β_2M) respectively. The sequence of each insert was confirmed by the Sanger sequencing method as described in section 2.4.8 (Sanger et al., 1977).

2.4.12 Maxi-preparation of Plasmids.

Plasmids for transfection experiments were prepared using an Endofree Plasmid Maxi Kit (Qiagen, UK). Basically, bacteria from a 300ml overnight culture were harvested by centrifugation (1200g) and the bacteria lysed for 5 mins at room temperature in 25mM Tris/HCl (pH8), 100mM NaOH, 0.5% SDS containing 10 IU/ml RNase. The preparation was neutralized with 1M potassium acetate (pH5.5) then filtered through a QIAfilter cartridge (Qiagen, UK). The filtrate was mixed with ER (Endotoxin Removal) buffer (Qiagen, UK) and incubated at 4^0C for 30 mins, then bound to an Anion Exchange Resin (Qiagen, UK) and set up in a column apparatus. After washing with a low salt buffer the plasmid DNA was eluted with a high salt buffer and then ethanol precipitated in the usual way. Finally, the plasmid DNA was dissolved in endotoxin free water. The preparation was stored at -20^0C until use.

2.4.13 In vitro-Translation Assay.

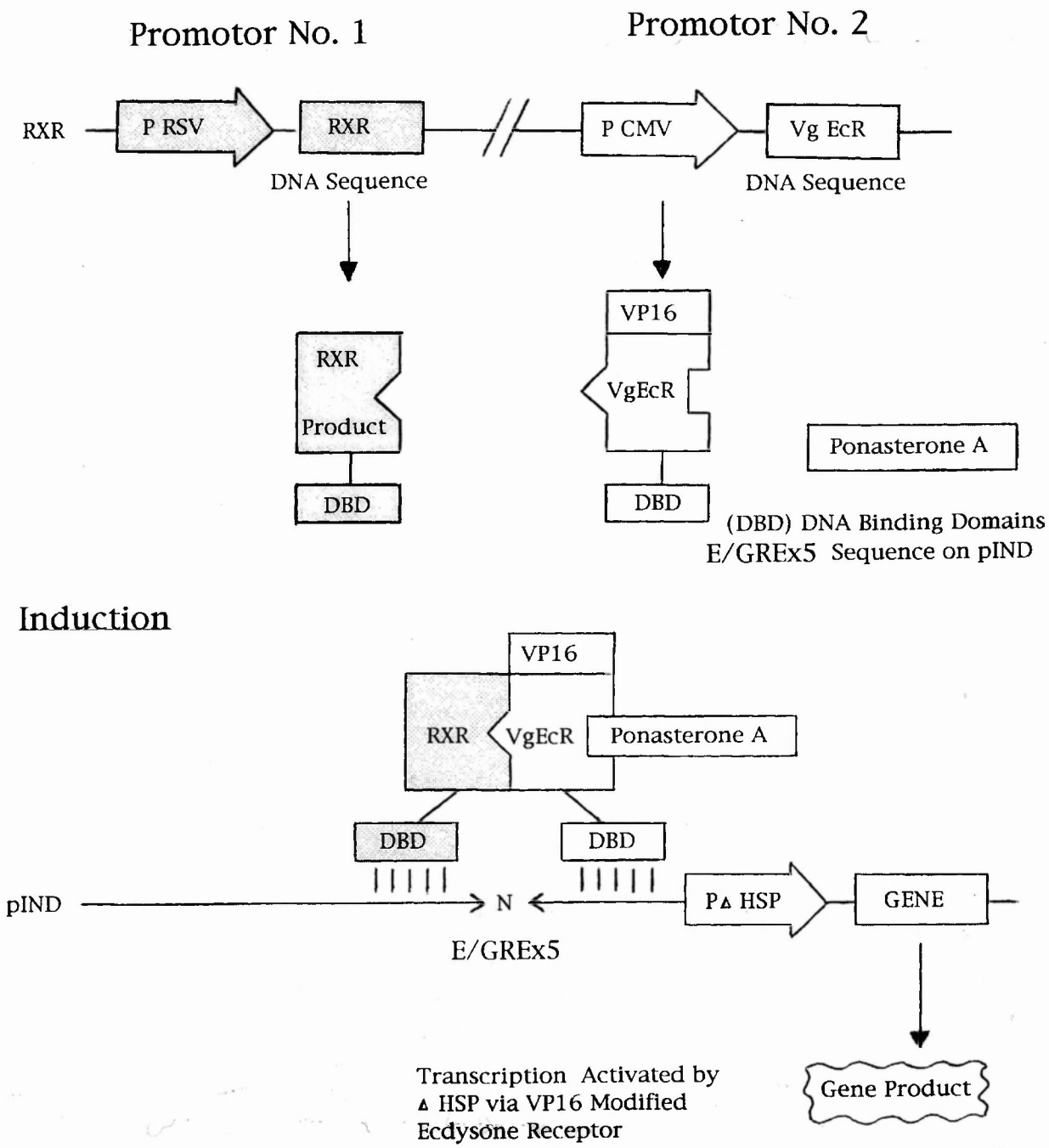
To confirm that the plasmid (pIND-β_2M15-A2) contained the β_2-microglobulin-linker-HLA-A2 construct, and that this insert coded for a protein of the expected size, an *in vitro*-translation assay was performed. The assay was based on the TnT Coupled Reticulocyte Lysate System (Promega, USA) and was performed according to the manufacturer's protocol. Briefly, a reaction mixture was assembled containing TnT Rabbit Reticulocyte Lysate, reaction buffer, amino acid mixture (minus Methionine), [^{35}S] Methionine (1,000Ci/mmol, Amersham, UK), RNasin (Ribonuclease Inhibitor) and template in the form of plasmid (pIND-β_2M15-A2). The reaction was performed in a total volume of 50µl at 30°C for 90 minutes. An aliquot (10µl) of the reaction was mixed with an equal volume of 2x reducing sample buffer (0.1M Tris/HCl pH6.8, 20% glycerol, 4% SDS, 2mM DTT and 0.001% Bromophenol Blue) and boiled for 10 minutes. The sample was electrophoresed in a 12.5% polyacrylamide/SDS gel (Laemmli, 1970). The gel was then dried down and bands visualized by autoradiography using Hyperfilm (Kodak, UK).

2.4.14 Transfection and Induction.

The cell lines, DLD-1 and CHO.KI, were maintained as described in section 2.1 above. DLD-1 cells (10^6) were co-transfected with either (pIND-β_2M) or (pIND-β_2M15-A2) (1µg) and (pVgRXR) (1µg) (Invitrogen, UK), using the Lipofectamine Plus system (Gibco/BRL) according to the manufacturer's protocol. [pVgRXR expresses the heterodimeric ecdysone receptor necessary in the inducible system illustrated in Figure 5]. The transfection reaction was allowed to continue overnight (17 hours) and then the cells were passaged by trypsinization and set up in fresh 90mm dishes at $2.5x10^4$ cells/ml. Stable co-transfectants of cells containing integrated DNA from both plasmids was achieved in E4 culture

FIGURE 5

An illustration of the Ecdysone inducible expression system.



The RXR construct, containing promotors 1 and 2, once transfected is continuously expressing the RXR and VgEcR proteins, each of which has a DNA binding domain. At the time of stimulation with Ponasterone A, these two DNA binding domaind are able to bind to the appropriate sequences on the pIND, upstream of the HSP promotor, allowing transcription of the required gene/construct to proceed.

medium supplemented with Geneticin (G418) at 1.5mg/ml and Zeocin at 100μg/ml.

A similar protocol was employed to transfect either (pIND-β_2M) or (pIND-β_2M15-A2) and (pVgRXR) into the cell line CHO.K1, however, this line was maintained in Iscove's modified Dulbecco's medium as described above in section 2.1. For dual selection of CHO.KI co-transfectants, G418 was used at 1mg/ml and Zeocin at 100μg/ml.

Co-transfections of both cell lines used were maintained in selection medium for 10-12 days at which time visible clones were picked and transferred to individual culture wells. When sufficient cells had grown, aliquots were set up in quadruplicate in microtitre plates. Duplicate wells were stimulated with Ponasterone A, 1μM (Invitrogen, UK), in appropriate culture medium for 24 hours. Control cells, in duplicate, were incubated in culture medium only. Microtitre plates were then screened for positive clones using an ELISA assay as described in section 2.3.2.

2.4.15 Magnetic Bead Selection of Tranfectants.

Selection of colorectal cell line DLD-1 that had been co-transfected, using the inducible Ecdysone system with either (pIND-β_2M) or (pIND-β_2M15-A2), was attempted using a magnetic bead attachment protocol. Expression of the transfected construct was induced with Ponasterone A, as described above in section 2.4.14. After 24 hours the cells were harvested by trypsinization and cells washed at 4^{o}C in DMEM containing 1% heat inactivated FCS. A 1:2 dilution of supernatant from antibody L368 (anti-$\beta_2$microglobulin) was added and the cells incubated for 30 mins at 4 ^{o}C, then washed twice at 4 ^{o}C, with DMEM containing 1% heat inactivated FCS. Following centrifugation the cell pellet was mixed with 200μl of a sterile, pre-washed, suspension of affinity purified goat-

anti-mouse IgG coated magnetic beads (M-450, Dynal, UK), $5x10^6$ beads/$1x10^6$ cells, and incubated for 30 mins at 4 ^oC. 2ml of DMEM/1% heat inactivated FCS was then added and the tube containing the cell/bead suspension placed in a magnet holder. The magnetic beads with attached cells were allowed to migrate towards the magnet which then allowed the unattached cells, in suspension, to be removed by pipetting. This magnetic separation process was repeated twice more. The resulting pellet of magnetic beads with attached cells was dispersed in 5ml of culture medium and set up in a culture flask. After incubating for 7 days most of the beads had became detached from the viable cell population and were removed from the flask during routine culture medium changes.

2.4.16 Cloning by Limiting Dilution.

Clones that grew out in selection medium in the primary transfection culture see 2.4.14 above, were hand picked and tested for their responsiveness to stimulation by Ponasterone A and expression of the transfected construct. Once such a clone had been identified it was subjected to single cell cloning by limiting dilution. To achieve this the adherent cells in culture were trypsinized as described above. After washing the cells in culture medium they were counted using a haemocytometer and diluted, in culture medium, to obtain a cell suspension of $2x10^1$per ml. Then 100µl of this cell suspension was then added to each of 32 wells in a 96 well flat bottom microtitre plate. The cell suspension was further diluted 1:1 and a similar volume added to the next 32 wells of the microtitre plate. Finally, the cells were diluted 1:1 again and 100µl added to each of the remaining 32 wells of the plate. The aim of this method was to plate out, on average, 2, 1 and 0.5 cells per microtitre plate well. Plates were then incubated in standard conditions and fed every seven days. When the cultures were approximately 50% confluent each clone was trypsinized and transferred to larger plates. Single cell cloning was achieved when approximately 1/3 of the wells, in one of cell dilution regions in the microplate, were seen to be growing healthy clones.

2.5 BIOCHEMICAL TECHNIQUES

2.5.1 Cell Lysis.

Cultured cells were harvested and counted then lysis performed at $5x10^6$/ml in ice cold lysis buffer (150mM NaCl, 1% NP40, 0.5% Deoxycholate, 0.1% SDS, 50mM Tris pH7.5, PMSF (100µg/ml) and Aprotinin, Leupeptin and Pepstatin-A all at 1µg/ml) for 30 mins at 4°C. The cell lysates were first frozen and thawed then sonicated for 1 minute. Finally, the lysates were centrifuged at 16,000g for 15 mins at 4°C to remove any cell debris. Aliquots of cell lysate were frozen at –70°C until required.

2.5.2 SDS PAGE and Western Blotting.

Aliquots of cell lysate, usually 100µl (equivalent to $0.5x10^6$ cells) were added to an equal volume of 2x reducing sample buffer (0.1M Tris/HCl pH6.8, 20% glycerol, 4% SDS, 2mM DTT and 0.001% Bromophenol Blue) and boiled for 10 minutes. Samples were electrophoresed on 8.5 - 18.5% polyacrylamide/SDS gels (Laemmli, 1970) then transferred on to Hybond nitrocellulose blotting membrane (Amersham, UK) using standard Western Blotting techniques (Towbin et al., 1979). Transfer was carried out for 17 hours at 4°C with 30V. Unreactive sites on the nitrocellulose were blocked by incubating in Phosphate buffered saline (PBS) containing 5% low fat dried skimmed milk (Tesco, London, UK). The membrane was then incubated in an appropriate dilution of mouse monoclonal antibody for 1 hour at room temperature, followed by five washes in PBS/5% low fat dried skimmed milk. Rabbit anti-mouse/Horse radish peroxidase conjugate was added at 1:10,000 dilution and incubated for 1 hour at room temperature. After 5 washes in PBS/5% low fat dried skimmed milk, the blot was developed using Enhanced Chemiluminescence (ECL, Amersham, UK) according to the manufacturers instructions and exposed to Hyperfilm (Kodak, UK).

2.5.3 Isoelectric Focussing.

Isoelectric focussing was used to discriminate HLA molecules of similar molecular weight that could not be resolved using conventional SDS/PAGE. The basic method is described in the 1987 International Histocompatibility Workshop (Yang, 1987). Cells were metabolically labeled with [^{35}S]Methionine (1,000Ci/mmol, Amersham, UK) in Methionine free Dubecco's modified Eagles medium, supplemented with 10% dialyzed, heat inactivated FCS for 17 hours. Cells were then harvested, washed once with PBSA, then lysed in a lysis buffer consisting of 150mM NaCl, 1% NP40, 0.5% Deoxycholate, 0.1% SDS, 50mM Tris pH7.5, PMSF (100µg/ml). The lysate was pre-cleared, by incubating for 1 hour at 4°C, with a preparation of washed Pansorbin A (Calbiochem, California, USA). HLA class I products were then immunoprecipitated by incubating the lysate for 1 hour at 4°C with HLA class I specific antibody W6/32, followed by isolation with Pansorbin A. Finally, the immunoprecipitates were incubated with Neuraminidase (Sigma Aldrich, UK) for 1 hour at 4°C. Samples were resuspended in sample buffer (0.1M Tris/HCl pH6.8, 10% glycerol, 2% SDS, 1mM DTT and 0.001% Bromophenol Blue) and boiled for 10 minutes prior to loading on a 30% acrylamide gel containing ampholines, pH3.5-10. pH5-7 and pH6-8 (Sigma Aldrich, UK). Gels were run for 17 hours at 10mA. The gel was then fixed in 10% acetic acid, washed and treated with Amplify (Amersham, UK) followed by drying. Bands were detected by autoradiography using X-ray film with an overnight exposure.

CHAPTER 3

RESULTS

3.1 Screening for Mutations in the β_2-microglobulin Gene.

3.1.1 Colorectal Cancer Cell Lines.

DNA was extracted from 52 established colorectal carcinoma cell lines and examined for mutations in the leader peptide/exon 1 region and exon 2 of the β_2-microglobulin gene. The leader peptide/exon 1 region and exon 2 were analyzed separately; PCR products were produced using the series of oligonucleotide primers and conditions described in Materials and Methods, section 2.4.2. Samples of these PCR reactions were then labeled with ^{32}P and analyzed by the SSCP technique using different conditions for electrophoresis of the amplicons as described in Materials and Methods, section 2.4.4. Eight of the 52 colorectal cell lines examined by SSCP showed band shifts, 5 in the leader peptide/exon 1 region and 3 in exon 2, as shown in Table 4. An example of the band shifts seen during SSCP is shown in Figure 6.

All the samples demonstrating a band shift were sequenced using PCR products as templates, in the direct solid phase sequencing system (Hultman et al., 1990), the protocols for template preparation and sequencing, performed by the Sanger method (Sanger et al., 1977), are described in Materials and Methods, section 2.4.8. The overall results are summarized in Table 5. Lovo is homozygous for a CT deletion in an 8 bp $(CT)_4$ repeat sequence located in the leader peptide/exon 1 sequence as shown in Figure 7. The pair of cell lines GP2d and GP5d, which are

TABLE 4

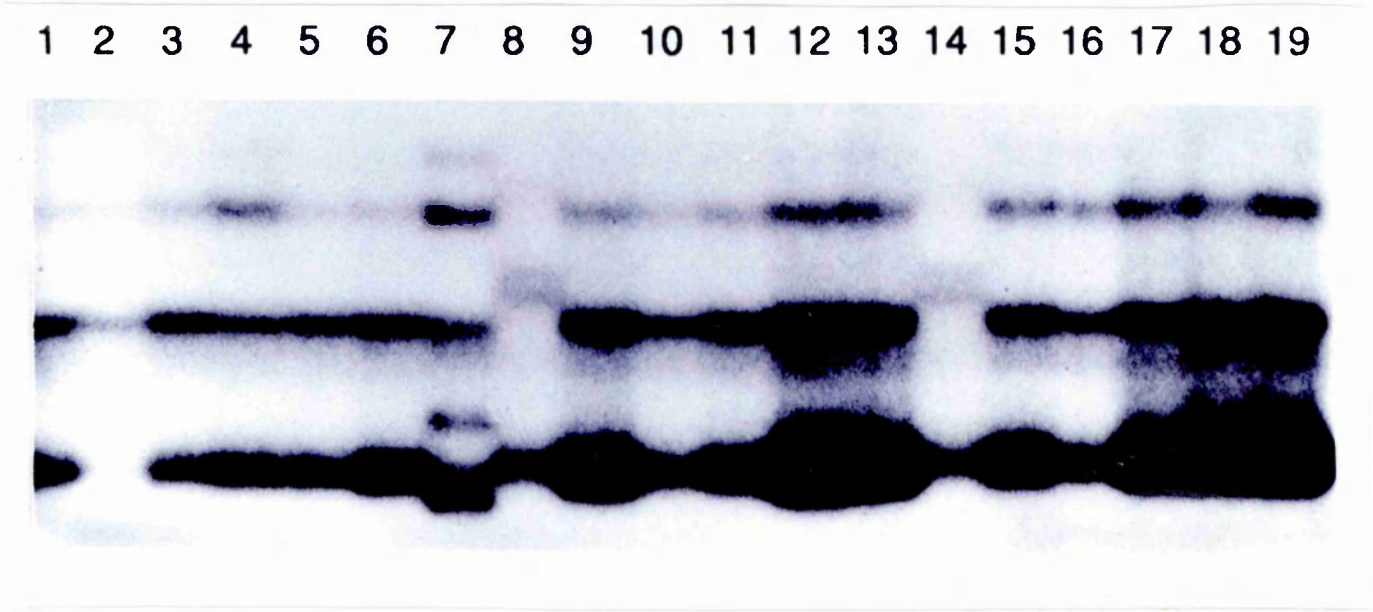
The panel of colorectal cancer cell lines studied showing the location of mutations found in the β_2-microglobulin gene.

(Figures in brackets indicate cell lines derived from the same individual.)

Cell Line		β_2-Microglobulin Mutation
T84		-
VACO 5		-
VACO 4S	1	-
VACO 4A	1	-
VACO 10MS		-
HRA19		Leader peptide/exon 1
SW 948		-
SW 837		-
SW 620	2	-
SW 480	2	-
SW 48		2 muts: Lead. pep/ex 1 & ex 2
SW 403		-
SW 1417		-
SW 1222		-
SKCO-1		-
PC/JW		-
LS 180	3	-
LS 174T	3	-
LS 1034		-
LoVo		Leader peptide/exon 1
CX-1	4	-
HT 29	4	-
WIDR	4	-
HCT 116		-
HCA 7		-
HCA 46		-
DLD-1	5	2 mutations in exon 2
HCT-15	5	2 mutations in exon 2
C125PM		-
COLO 320DM		-
COLO 206	6	-
COLO 205	6	-
COLO 201	6	-
CC20		-
CCO7		-
CACO 2		-
C 99		-
C 84		Exon 2
C 80		-
C 75		-
C 70		-
C 32		-
C 106		-
C 10		-
SW 1116		-
GP2d	7	Leader peptide/exon 1
GP5d	7	Leader peptide/exon 1
LS 411		-
COLO 741		-
HT 55		-
LIM 1863		-
COLO 678		-

FIGURE 6

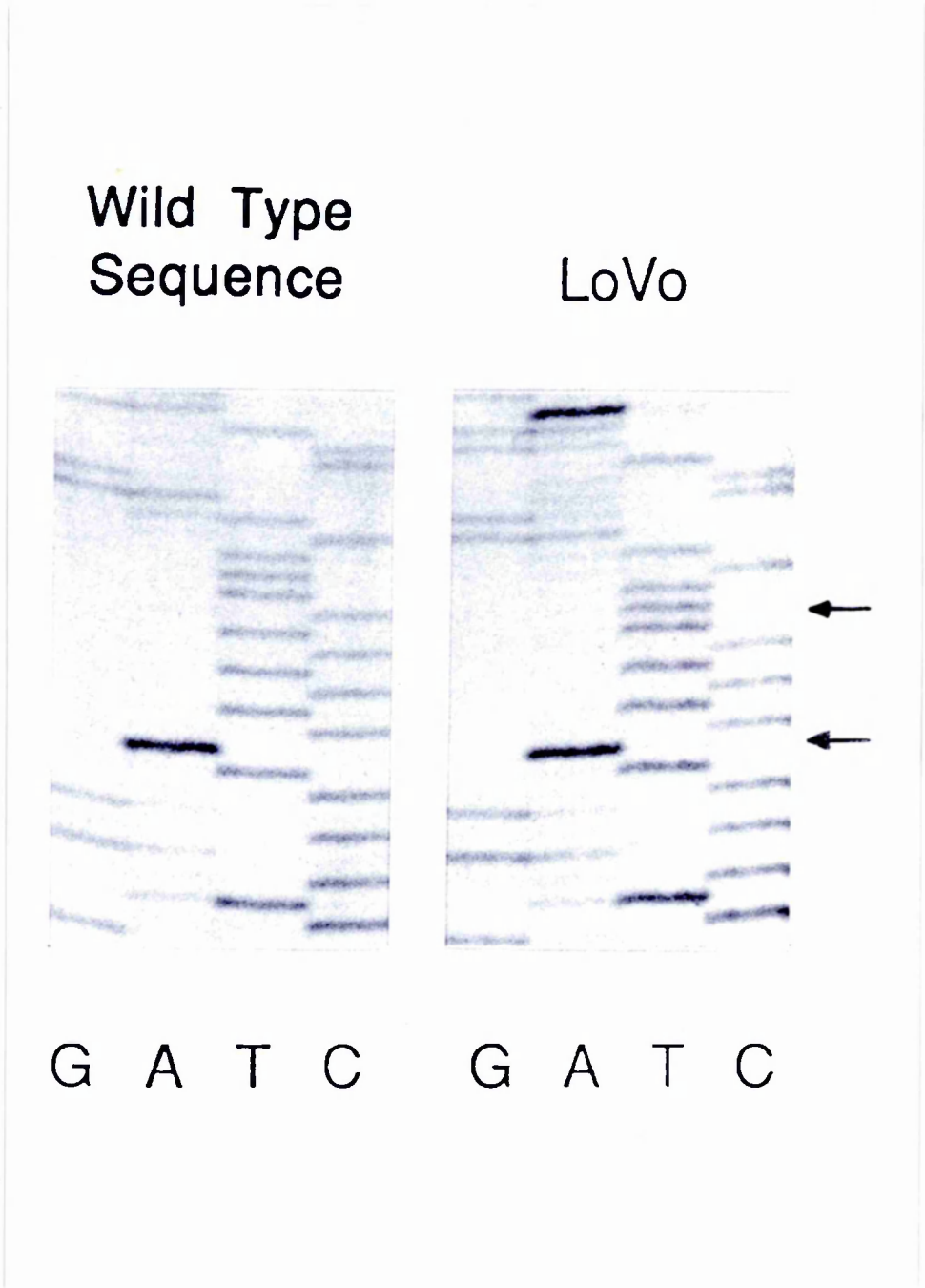
Autoradiograph of SSCP analysis of $\beta_2$microglobulin leader peptide/exon 1 PCR products from a series of colorectal cancer cell lines.



DNA was amplified using primers 32892 and 32894 as described in Materials and Methods section 2.4.2 and analysed by SSCP as described in section 2.4.4. Products were denatured and electrophoresed on an 8% polyacrylamide gel containing 17% glycerol. Lanes: 1, T84; 2, VACO 5; 3,SW948; 4, SW837; 5, SW403 ; 6, SW1222 ; 7, HRA19 ; 8, LoVo ;9, PC/JW ;10 HCA46 ; 11, C80; 12, C70; 13, CACO2 ; 14, GP2d; 15, HT55; 16, C106; 17, WIDR; 18, SKCO1; 19, CC20.

FIGURE 7

Autoradiograph from a sequencing gel of $\beta_2$microglobulin leader peptide/exon 1 PCR products from a wild-type control and the colorectal cancer cell line LoVo.



Single strands were isolated from PCR products using magnetic bead technology and sequenced by the 'Sanger" method as described in Materials and Methods, section 2.4.8. Samples were: a normal lymphoblastoid B cell line (Left panel) showing wild-type sequence, and LoVo (right panel) showing a CT -> deletion in the 8 bp repeat region. This region is indicated by the arrows.

derived from the same patient, both exhibit homozygous frame shifts, similar to that identified in LoVo, in the $(CT)_4$ repeat element of the leader peptide/exon 1 sequence. SW-48 and HRA-19 show different 4-bp deletions (CTCT for SW-48 and TCTT for HRA-19) in the same region of the leader peptide sequence, however, the presence of the wild-type sequence at these positions indicated that these cell lines were heterozygous for their respective mutations. For control purposes, the Burkitt lymphoma cell line, Daudi, was sequenced and shown to have a homozygous G->C mutation in the initiation ATG of the leader peptide sequence (data not shown) and was in agreement with data reported earlier (Rosa et al., 1983).

The colorectal cell line C84 has a heterozygous G->A point mutation in codon 32 of exon 2 (see Table 5 and Figure 8) changing an Asp to an Asn. The cell lines DLD-1 and HCT-15, which are derived from the same patient, both demonstrate two mutations in exon 2 as shown in Figure 9. A point mutation (G->A) was found at the last base of intron 1 (between exon 1 and exon 2), and a C->A point mutation in codon 10 changes a Try into a stop codon.

The colorectal cell lines that were demonstrated by sequencing to have a mutant β_2-microglobulin gene were examined for expression of β_2-microglobulin by ELISA assay using a panel of monoclonal antibodies. The cell lines SW-48, GP2d, GP5d, DLD-1 and HCT-15 showed no reactivity with antibody W6/32 (which recognizes mature HLA-A, B and C complexed with β_2-microglobulin) and the absence of free β_2-microglobulin chains was confirmed in these cell lines by the lack of reactivity with the β_2-microglobulin specific antibodies L368 and BBM.1, see Table 6. LoVo has previously been shown to be deficient in β_2-microglobulin expression (Brodsky et al., 1979b). HRA-19 and C84 are both heterozygous for different single β_2-microglobulin mutations and show intermediate levels of β_2-microglobulin expression with antibodies W6/32 and BBM.1. However, HRA-19 demonstrates reduced reactivity with antibody L368 whilst C84 appears to have normal reactivity with L368. The colorectal cell line HT-29 is included in the data shown in Table 6 as a positive control.

TABLE 5

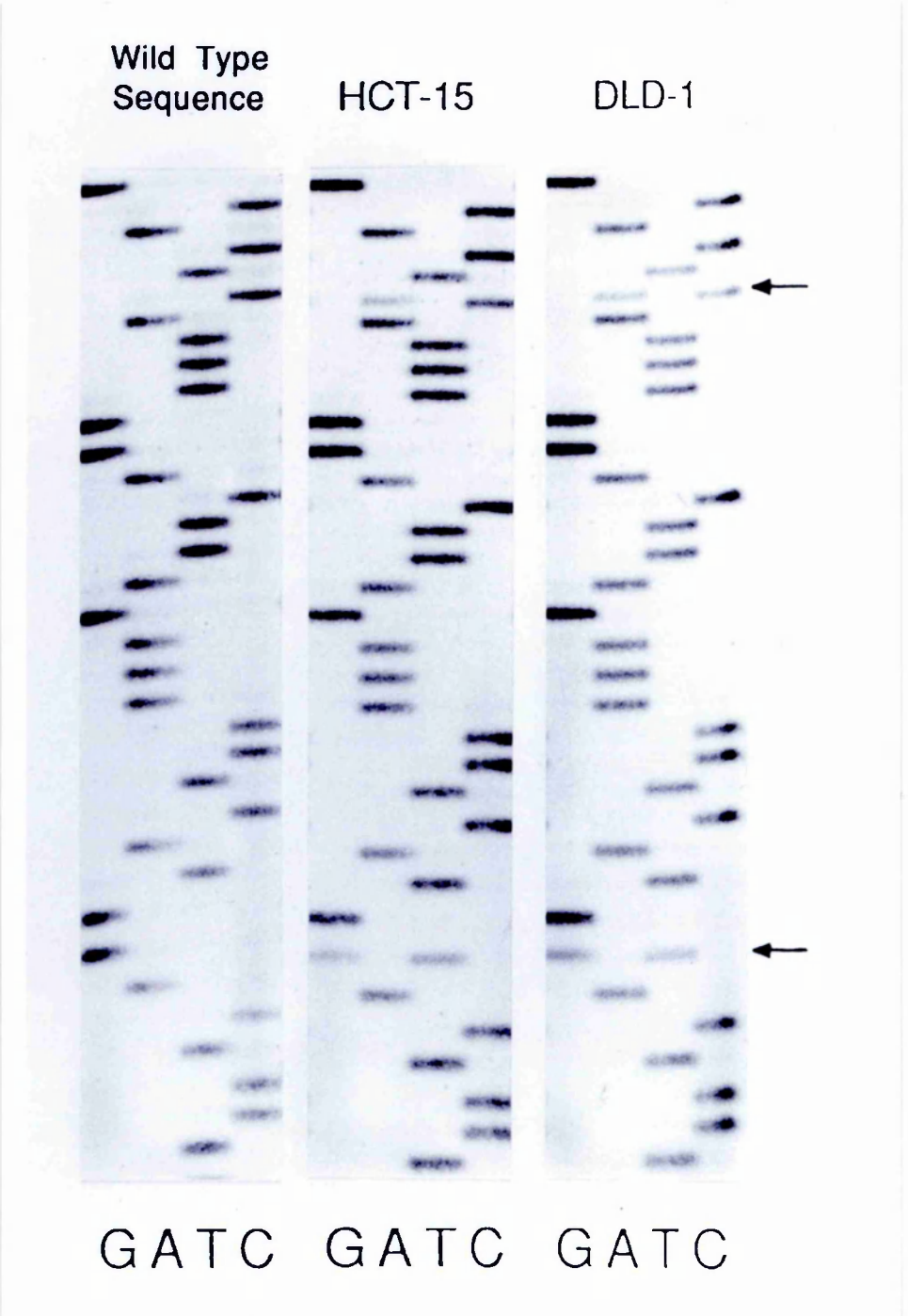
Mutations of β_2-microglobulin in colorectal cancer cell lines identified by SSCP and sequencing.

Cell Line	Exon	Homozygous/ Heterozygous	Mutation	Stop Codon
LoVo	Leader sequence	Homozygous	CT deletion Codons13-15+	at Codon36
HRA-19	Leader sequence	Heterozygous	TCTT deletion Codons 14&15+	at Codon23
GP2d/ GP5d	Leader sequence	Homozygous	CT deletion Codons 13-15+	at Codon 36
SW-48	Leader sequence	Heterozygous	CTCT deletion Codons 13-15+	at Codon 23
SW-48	2	Heterozygous	A deletion Codon 47	at Codon 82
C84	2	Heterozygous	G -> A Codon 32	Asp -> Asn
DLD-1/ HCT-15	2	Heterozygous	C -> A Codon 10	at mutation Codon 10
DLD-1/ HCT-15	2	Heterozygous	G -> T Last base in intron 1	No stop codon

+numbers represent codons in the Leader sequence.

FIGURE 9

Autoradiograph from a sequencing gel of $\beta_2$microglobulin exon 2 PCR products from a wild-type control and the two colorectal cancer cell lines HCT-15 and DLD-1 (derived from the same patient).



Single strands were isolated from PCR products using magnetic bead technology and sequenced by the 'Sanger" method as described in Materials and Methods, section 2.4.8. Samples were: a normal lymphoblastoid B cell line (Left panel) showing wild-type sequence; HCT-15 (Middle panel) and DLD-1 (right panel) that show two heterozygous mutations, C -> A (Upper arrow) and G -> T (lower arrow).

TABLE 6

HLA class I and β_2-microglobulin expression in colorectal cancer cell lines exhibiting a mutation in β_2-microglobulin, determined by ELISA.

Cell line	Mutation Location	Homozygous/ Heterozygous	Antibodies		
			W6/32	L368	BBM.1
HT-29	None		3389	4000	2857
LoVo	Leader seq.	Homozygous	*	*	*
HRA-19	Leader seq.	Heterozygous	923	2865	305
SW-48	Leader seq. & exon2	Both Heterozygous	141	184	492
GP2d	Leader seq	Homozygous	22	54	ND
GP5d	Leader seq	Homozygous	61	77	ND
HCT-15	2 mutations in Exon2	Both Heterozygous	0	0	370
DLD-1	2 mutations in Exon 2	Both Heterozygous	75	92	234
C84	Exon2	Heterozygous	1588	4000	1795

* Previously shown to to deficient for β_2-microglobulin (Travers , Arklie *et al.* 1982)

ND - Not Done

The ELISA was performed as described in Materials and Methods, section 2.3.2. Results shown are the readout from Microfluor plate reader, with a maximum reading of 4000.

3.1.2 Fresh Tumour Samples.

A collection of 280 tumour samples (147 Colorectal, 30 Melanoma, 48 Breast, 21 Ovarian and 34 Lymphoma) was assembled from various sources (as indicated in Materials and Methods, section 2.2) and DNA extracted from all samples and examined for mutations in the leader peptide/exon 1 region and exon 2 of the β_2-microglobulin gene. This was performed by PCR followed by SSCP as described above for the colorectal cell lines (section 3.1.1). The types of tumour examined and the frequency of β_2-microglobulin mutations identified are shown in Table 7. Nine of the 147 colorectal tumours in the collection exhibited β_2-microglobulin mutations, identified as band shifts during SSCP analysis. An example is illustrated in Figure 10. In contrast only one of the remaining 133 non-colorectal tumours examined contained a β_2-microglobulin mutation.

Samples demonstrating a band shift by SSCP were sequenced directly from PCR products. A magnetic bead technique was used to isolate single strands (Hultman et al., 1990) which were then sequenced by the Sanger method (Sanger et al., 1977), as described in Materials and Methods, section 2.4.8. In the colorectal tumours, six mutations were identified in the leader peptide/exon1 sequence; two in exon 2 and one tumour (C43) contained a point mutation at the base before the intiation ATG. Seven of the β_2-microglobulin mutations in the colorectal tumours occurred at repeated elements. A 2 bp deletion in the 8 bp $(CT)_4$ repeat region in the leader peptide sequence was found to be overrepresented in this group, ocurring in three of the seven, C14, C108 and 13971/92. These are similar to the frame shift deletions seen in the colorectal cell lines LoVo, GP2d and GP5d except that in the fresh tumour samples the wild type sequence is also present (see Figure 11 showing the sequence obtained for tumour C14). Tumour StM78 contained a minus (TG) frameshift in a $(TG)_2$ repeat sequence and 3624/91 showed a G->C point mutation in a $(CG)_2$ repeat, both these repeated sequences are located in the

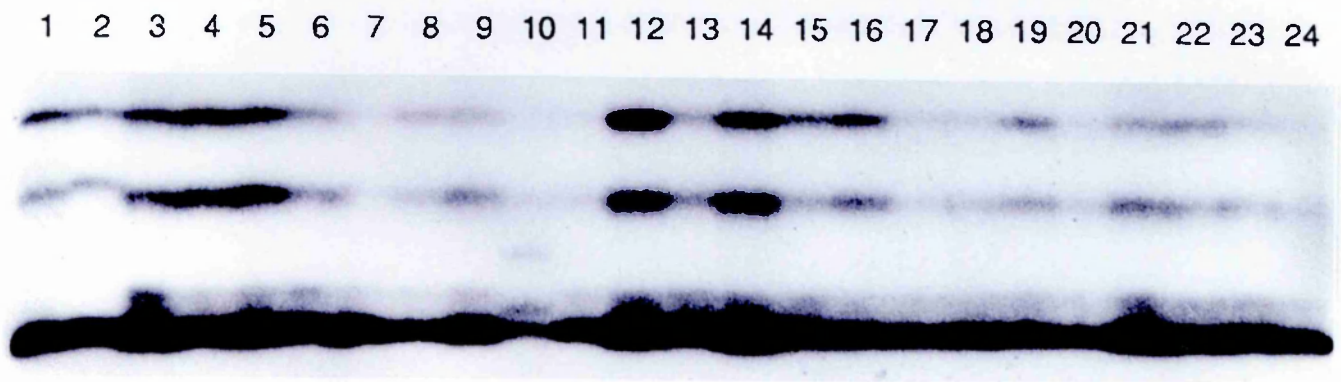
TABLE 7

β_2-microglobulin mutations in tumours from different sites.

Tumour	Number analyzed	Number with β_2-m Mutations
Colorectal	147	9
Melanoma	30	0
Breast	48	0
Ovary	21	0
Lymphoma	34	1
TOTAL	280	10

FIGURE 10

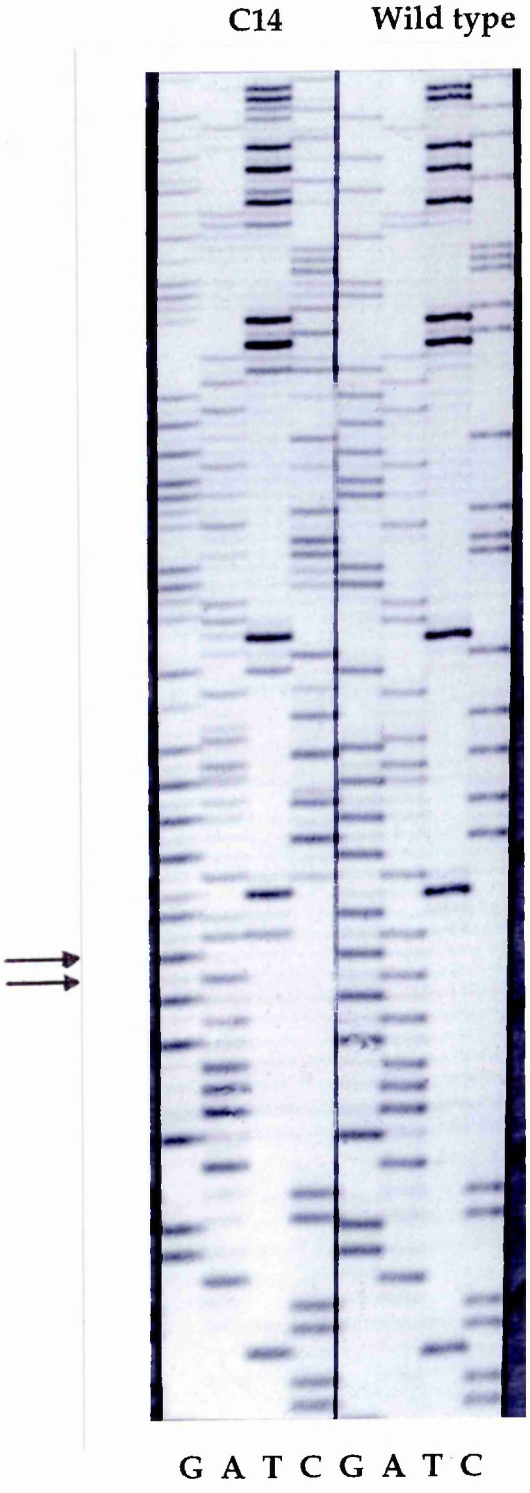
Autoradiograph of SSCP analysis of $\beta_2$microglobulin leader peptide/exon 1 PCR products from a series of fresh colorectal tumours.



DNA was amplified using primers 32892 and 32894 as described in Materials and Methods section 2.4.2 and analysed by SSCP as described in section 2.4.4. Products were denatured and electrophoresed on an 8% polyacrylamide gel containing 17% glycerol. Lanes: 1, Normal Lymphoblastoid B cell line (Bristol-8); 2, Colorectal cancer cell line LoVo (positive control); 3-24, a series of 22 fresh tumour samples.

FIGURE 11

Autoradiograph from a sequencing gel of $\beta_2$microglobulin Leader peptide/exon 1 PCR products from a wild-type control and fresh colorectal tumour C14.



Single strands were isolated from PCR products using magnetic bead technology and sequenced by the 'Sanger" method as described in Materials and Methods, section 2.4.8. Samples were: a normal lymphoblastoid B cell line (right panel) showing wild-type sequence and C14 (Left panel) showing a Heterozygous CT deletion (indicated by the arrows) in the 8 bp CT repeat region in the leader peptide sequence.

TABLE 8

Mutations of β_2-microglobulin in fresh colorectal tumours identified by SSCP followed by sequencing.

SAMPLE	LOCATION	MUTATION	SITE
C14	Leader sequence	CT deletion	(CT)4 Bases 37-44*
C43	Base before ATG mutation	G -> A point upstream of ATG	(GA)2
C84T	Exon 2	G -> A point Mutation	Non repetitive sequence
C108	Leader Sequence	CT deletion	(CT)4 Bases 37-44*
StM78	Leader sequence	TG deletion	(TG)2 Bases 24-27*
StM185	Exon 2	A deletion	(A)5 Codons 47/48+
3624/91	Leader sequence	G -> C point mutation	(CG)2 Bases 30-33*
3822/93	Leader sequence	CT -> GG	Non repetitive sequence
13971/92	Leader sequence	CT deletion	(CT)4 Bases 37-44*

* Numbers represent base position in the Leader sequence
+ Codon number

TABLE 9

Expression of β_2-microglobulin in fresh colorectal tumours exhibiting a β_2-microglobulin gene mutation.

Tumour Number	Mutation location	β_2-microglobulin expression*
C14	Leader seq/exon1	Low**
C43	Base before ATG	Low**
C84T	Exon 2	Low**
C108	Leader seq/exon 1	Low**
3624/91	Leader seq/exon 1	Not detectable
3822/93	Leader seq/exon 1	Not detctable
13971/92	Leader seq/exon 1	Not detectable
StM78	Leader seq/exon 1	N/D+
StM185	Exon 2	N/D+

* Immunocytochemical detection using antibodies W6/32 and BBM.1 as described in Materials and Methods, section 2.3.4. Tumour expression was determined by comparing the intensity of staining to that of lymphocytes and surrounding stromal cells.

**Low reactivity implies heterozygous β_2-microglobulin mutation.

+ Not Done

leader peptide sequence. Tumour (C43) contained a G->A point mutation in the base immediately preceding the ATG initiation codon and also occurred in a $(GA)_2$ repetitive sequence. StM185 contained a single A deletion in an $(A)_5$ repeat located in exon 2. Only two mutations were seen in non-repetitive sequences, in tumour 3822/91 (CT->GG change in the leader peptide/exon 1 region) and C84T (G->A point mutation in exon 2), see Table 8. In the group of non-colorectal tumours, one mutation was found in a lymphoma, which was localized to the leader peptide/exon 1 region, but was not further sequenced or investigated.

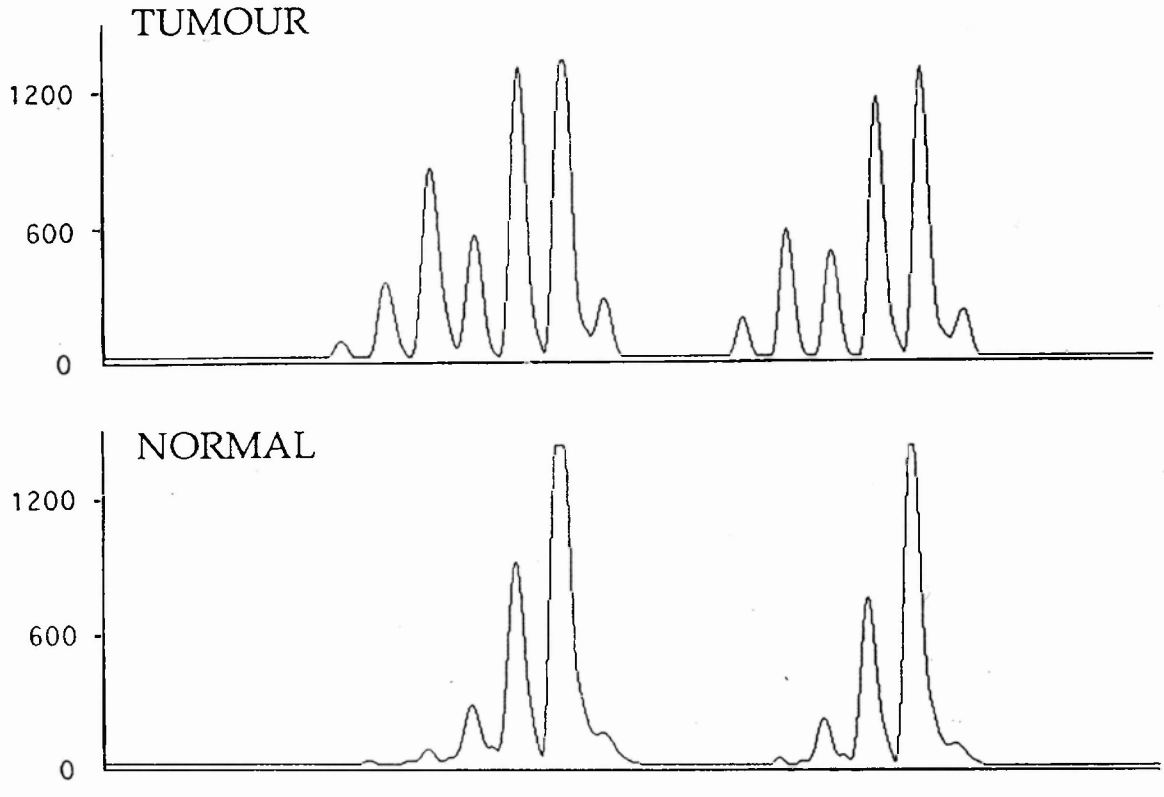
Seven of the nine mutated tumours were examined by immunocytochemistry with antibodies W6/32 and BBM.1, which recognize mature HLA-A, B and C complexed with β_2-microglobulin and free β_2-microglobulin chains, respectively. In all the cases examined the expression of β_2-microglobulin was reduced in comparison to that in adjacent normal stromal cells and lymphocytes present in the section. In three cases, β_2-microglobulin was undetectable, see Table 9, suggesting that these tumours could be homozygous for their respective β_2-microglobulin mutations.

3.2 Microsatellite Analysis.

Microsatellite instability can be used to detect defects in DNA mismatch repair. From the panel of colorectal cell lines 40 were analyzed at three or more CA repeat microsatellite markers as described in Materials and Methods (Section 2.4.7). Loci used were taken from the 1993-4 Genethon human genetic linkage map and include D2S119, D2S123, D2S378 (chromosome 2), D10S197, D10S209 and AFMb001 (chromosome 10), DS13175 (chromosome 13) and D17S941 (chromosome 17) (Gyopay et al., 1994). Those cell lines showing evidence of alterations at two or more microsatellites were considered to exhibit microsatellite instability. In addition, a sub-set of 71 fresh colorectal tumours was analyzed at three or more marker loci and, in 37 of these cases normal DNA was available which allowed a more accurate assessment of microsatellite instability by the comparison of peak patterns obtained for normal and tumour DNA as shown in Figure 12.

FIGURE 12

Microsatellite analysis of a colorectal tumour sample and normal epithelium from the same patient, using di-nucleotide repeat marker D2S123.



PCR was performed using the appropriate primer pair for D2S123, with the 5' primer fluorecently tagged. The PCR conditions are described in Materials and Methods, section 2.4.7. Dilutions of PCR products were made in denaturing buffer and analyzed in a 6% polyacrylamide denaturing gel on an ABI 377A Genotyper. Samples were: Upper panel, DNA from Fresh tumour C108; Lower panel, DNA from Normal colon epithelia from patient C108.

TABLE 10

Microsatellite status of the colorectal cancer cell lines and fresh colorectal tumour samples that exhibited a β_2-microglobulin mutation.

	Sample	β_2-Microglobulin mutation	Microsatellite status*
CELL LINES			
	LoVo	+	Unstable
	DLD-1 (1)	+	Unstable
	HCT-15 (1)	+	Unstable
	SW48	+	Unstable
	C84	+	Unstable
	GP2d (2)	+	Unstable
	GP5d (2)	+	Unstable
	HRA-19	+	ND**
	HCA-7	-	Unstable
	LS174T	-	Unstable
	LS411	-	Unstable
FRESH TUMOURS			
	C14	+	Stable
	C43	+	Stable
	C84T	+	Unstable
	C108	+	Unstable
	StM78	+	Stable
	StM185	+	Unstable
	3624/91	+	Stable
	3822/93	+	Unstable
	13971/92	+	Unstable

*Microsatellite status determined by instability at two or more loci. For details see Materials and Methods, section 2.4.7.

** ND – Not Done

(1) DLD-1 and HCT-15 are derived from the same patient
(2) GP2d and GP5d are derived from the same patient

Ten of the 40 colorectal cell lines analyzed were judged to have multiple peaks, at two or more marker sites, and were called microsatellite unstable. Seven of these cell lines contain mutations in the β_2-microglobulin gene and three contained no β_2-microglobulin mutations, see Table 10.

Seventy one DNA samples, from fresh colorectal tumours and including the 37 samples where corresponding normal material was available were analyzed. Seven (10%) showed evidence of alterations at two or more microsatellite loci and were considered to exhibit microsatellite instability. The microsatellite status of the fresh colorectal tumours that contained a mutation in the β_2-microglobulin gene is shown in Table 10.

3.3 A mutation in the HLA-A*0101 gene found in the colorectal cell line HCA-7.

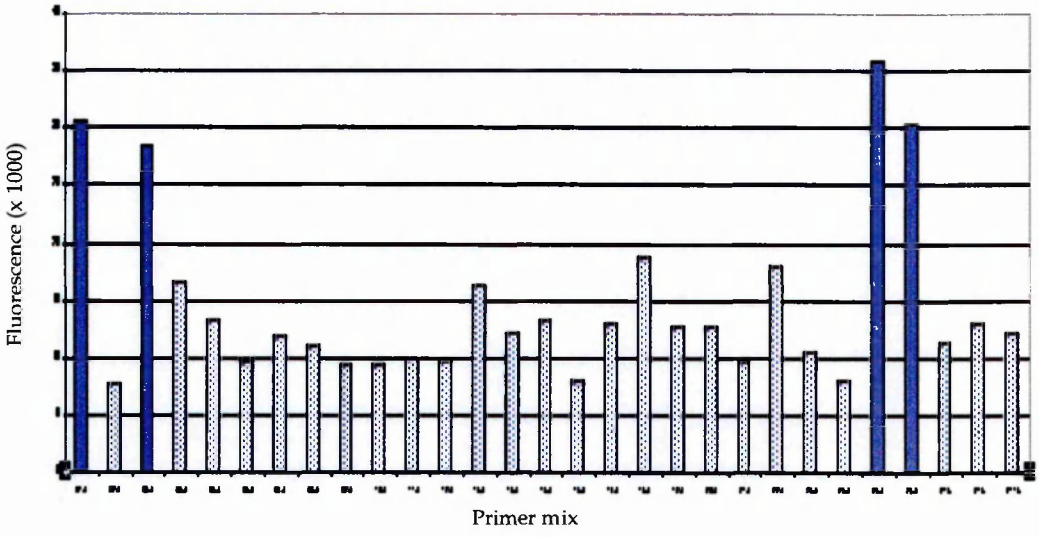
The colorectal cell line, HCA-7, and the normal B lymphoblastoid cell line, EVA-1224 (derived from the same individual) were HLA class I typed at the DNA level using an ARMS-PCR based assay, followed by detection using the AMDI technique, as outlined in Materials and Methods section 2.4.6. A series of 96 allele specific primers was used for typing and the results shown in Figure 13(A&B). At the HLA-A locus HCA-7 and EVA-1224 both type as -A*0101, -A*0201 and both have the same alleles at the HLA-B and C loci, see Figures 13(A&B).

To investigate HLA-A1 expression at the protein level, samples from the cell lines HCA-7 and EVA-1224 were subjected to isoelectric focusing. Cultures from these cell lines were metabolically labeled with [^{35}S]Methionine for 17 hours as described in Materials and Methods, section 2.5.3. After harvesting and lysing the cells, lysates were pre-cleared by adsorption with Pansorbin A, then HLA class I products immunoprecipitated with antibody W6/32, followed by analysis on an isoelectric focusing gel containing ampholines to create a pH gradient. Bands were detected by autoradiography. As shown in Figure 14, HCA-7 and

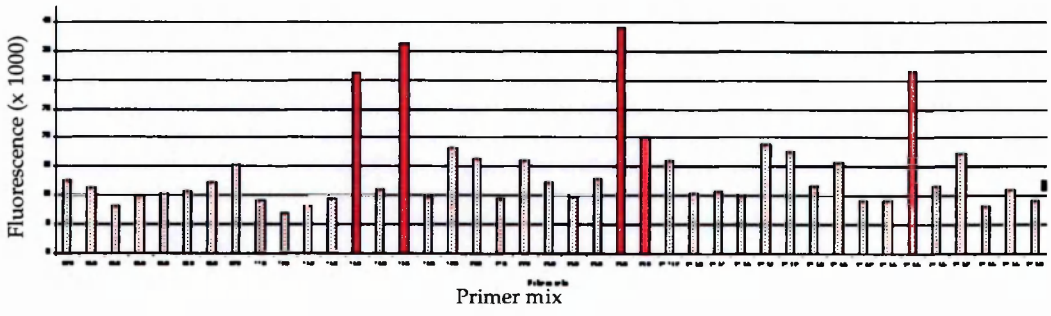
FIGURE 13A

HLA typing of normal Lymphoblastoid cell line EVA-1224 derived from the same patient as colorectal cancer cell line HCA-7

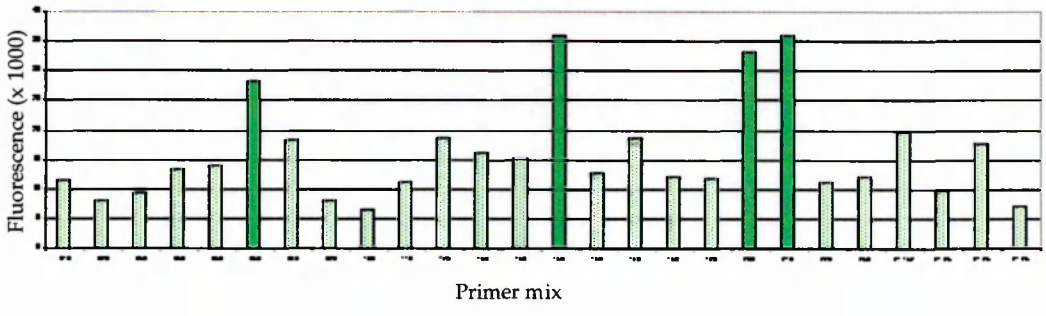
A Locus



B Locus



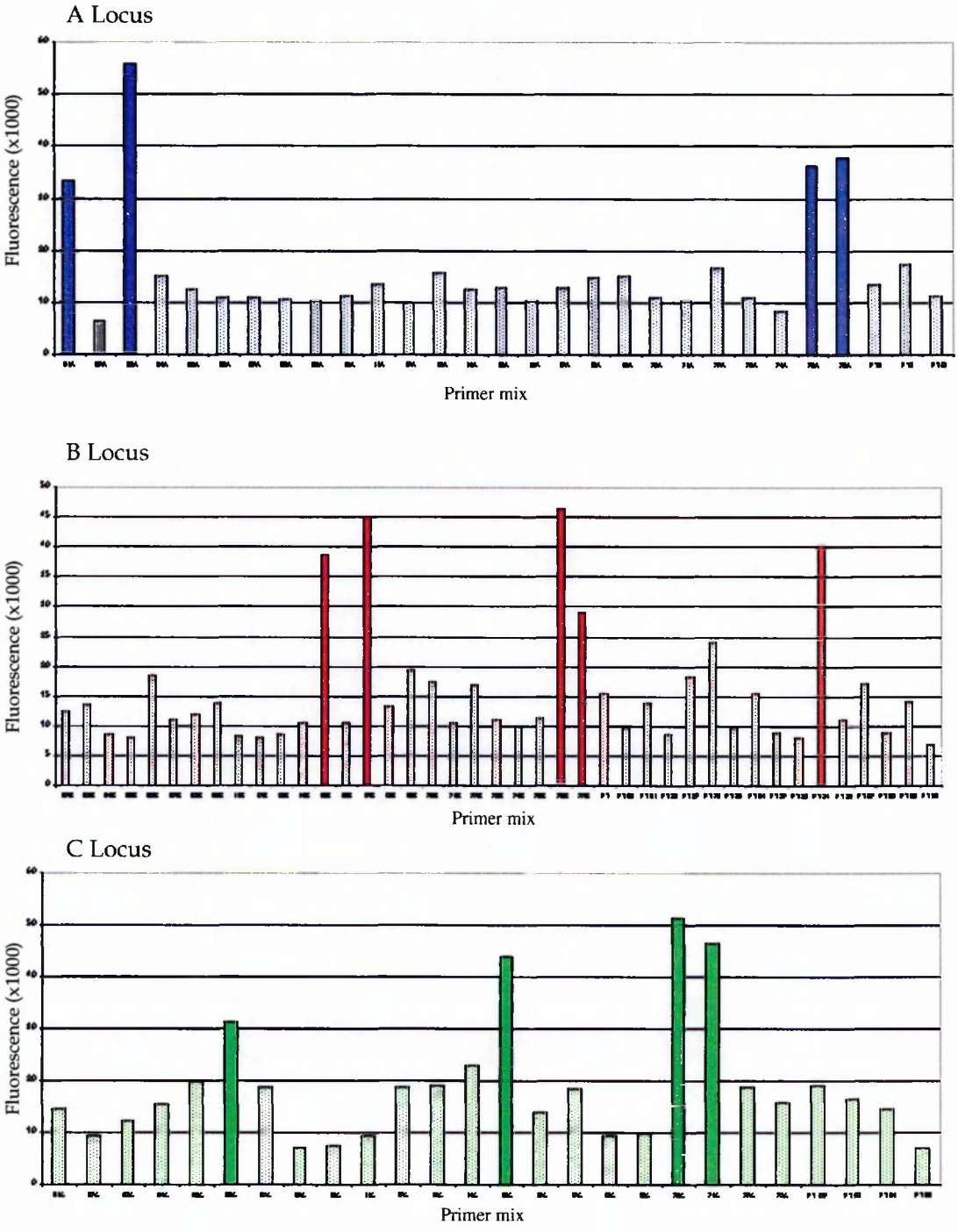
C Locus



For legend to Figure 13A, see page 78

FIGURE 13B

HLA typing of the colorectal cancer cell line HCA-7, derived from the same patient as Lymphoblastoid cell line EVA-1224.



For legend to Figure 13B, see page 78

FIGURE 13 A & B

HLA-A, B and C typing of cell lines EVA-1224 and HCA-7

Legend for Figures 13A and 13B

The HLA-A, B and C typing of the cell lines EVA-1224 and HCA-7 was performed from DNA samples from the cell lines using Sequence specific primers for each allele and performed as described in Materials and Methods, section 2,4,6 using ARMS-PCR followed by AMDI detection. The primers used and their specificities are shown below. Figure 13A shows the typing for EVA-1224 and Figure 13B the typing for HCA-7.

HLA-A Locus primers

MIX	LANE	SPECIFICITIES	approx SIZE
01A	1	A*0101/2/3/4N	574bp
02A	2	A*3601	563bp
03A	3	A*0201-22/24-27/29, *6802	513bp
04A	4	A*0301/2/3N/4, *2403v	626bp
05A	5	A*2301, *2405v/13	555bp
06A	6	A*2402-5/7/9N-11N/14	555bp
07A	7	A*2501/2	398bp
08A	8	A*2601/2/477/8/9/11N,*4301	400bp
09A	9	A*2501/2, *2601-4/6/9/11N,*2407/2, *6601-3	438bp
10A	10	A*2502,*3401/2,*6601/2	417bp
11A	11	A*4301	440bp
12A	12	A*1101/2/4	518bp
13A	13	A*6601/2/6/8,*6901	445bp
14A	14	A*6801/3/4/5/8,*3402	426bp
15A	15	A*6802	623bp
16A	16	A*6901	405bp
17A	17	A*2901/2/3	515bp
18A	18	A*3001-4/6	560bp
19A	19	A*31012	481bp
20A	20	A*3201/2	421bp
21A	21	A*33013	461bp
22A	22	A*3201/2,*7401-3	496bp
23A	23	A*2901/2/3,*31012,*3301/3,*7401/2	414bp
24A	24	A*8001	543bp
25A	25	A*2301-2/25-27/29,*2401-17/3/4/7/8,*2601-4/6,*6801-3	446bp
26A	26	All = eg: A*2301-2/25-27,*2401-17/3/4,*2601-4/6,*6801	446bp
PT5	27	A*2301,*2402-7/9N-11N/13/14	464 bp
PT8	28	A*2501/2, *2601/2/3/7/8/11N,*4301,*6601/3	170bp
PT13	29	A*2609,*3401/2	170bp

HLA-B Locus primers

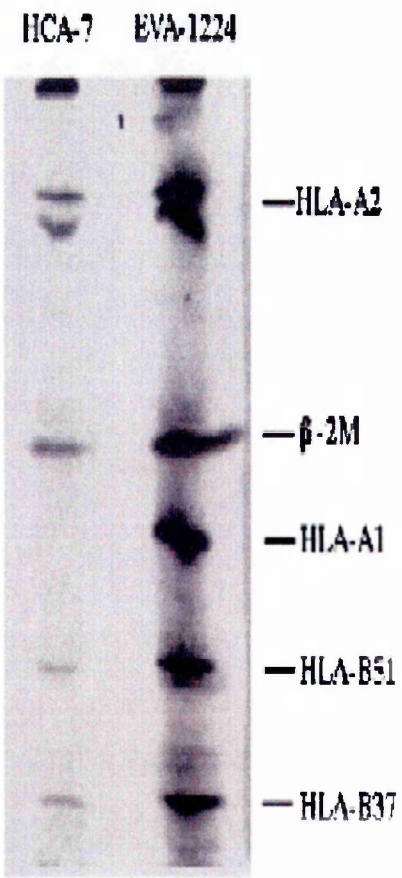
MIX	LANE	SPECIFICITIES	approx SIZE
02B	1	B*4901-11/15-19,*4701/2/3	511bp
03B	2	B*4101/2/3,*4202	781bp
04B	3	B*4001/7/8/10/14-17	784bp
05B	4	B*1301/2	471bp
06B	5	B*4402-5/7/8/10	537bp
07B	6	B*4402-5/7-10,*1514,*4501,*5002	575bp
08B	7	B*4501,*4901,*5001/2	600bp
09B	8	B*5401,*5501-3/5,*5601/2/4,*5901,*8201,Cw*1502/3	490bp
11B	9	B*1401/2/3/4	390bp
12B	10	B*0702-5,*4008,*8101	619bp
13B	11	B*0801-3	606bp
14B	12	B*1801-5	609bp
15B	13	B*3701,*4406,*5108	606bp
16B	14	B*3501-9/11/13/17-19/21/23/24/25,*5301/2	390bp
17B	15	B*5101-9/11N/14,*5201,*7801/2/3	504bp
18B	16	B*5701-4	380bp
19B	17	B*5801	345bp
20B	18	B*1501(01-09/11-14/49)/24-27/35-41/42,*1504,*4801,*4804	422bp
21B	19	B*1502/5/6/8/9/10/12/13/16/17/26/35/38/39/46/48,*1504,*4501,*4808,*5101-3	623bp
22B	20	B*4201/2	702bp
23B	21	B*4001/10/12/14-17,*4801/3	608bp
24B	22	B*1509/10/18/21/23/37, *7803	562bp
25B	23	B*5401	506bp
26B	24	Bw4	180bp
27B	25	Bw6 not B*5401	180bp
PT172	26	B*3801/2	448bp
PT50	27	B*3901/0913-22/24,*6701/1012	507bp
PT51	28	B*6701/6702, *3910	548bp
PT58	29	B*4901,*5901	385bp
PT57	30	B*4901,*5001/2,*4005/15/16/17,*2704/6/10,	635 bp
PT79	31	B*3501-4/6-9/11/13/15/17/19/20/23/25-29/31-35/37/39/41/44/46/48,*5104,*5301/2	369bp
PT80	32	B*3501-13/15-18/20-24,*1522,*1801-5,*7801/2,	128bp
PT54	33	B*1513,*4406,*5104,*5301/2, *5705, *5801	319bp
PT62	34	B*1501/2/9,*7801/2/3,	400bp
PT63	35	B*5101-9/11N/14,*52011,*52012	401bp
PT64	36	B*5101-9/11/14,*7801/2/3,*1509	451bp
PT65	37	B*15012,*52011/52012	440bp
PT52	38	B*2701-11	1401/200bp
PT66	39	B*7301	289bp
PT55	40	B*3702, **4701/2/3	414bp
PT60	41	B*5601/2/3/4,*6201	551bp

HLA-C Locus primers

MIX	LANE	SPECIFICITIES	approx SIZE
01C	1	Cw*0102	1026bp
02C	2	Cw*0202/3,*1701/2	521bp
03C	3	Cw*0302-6/8/9	563bp
04C	4	Cw*0401-5, *1801/2	330bp
05C	5	Cw*0501	563bp
06C	6	Cw*0602/3/4	304bp
07C	7	Cw*0701-3/6	1062bp
09C	8	Cw*0303	530bp
10C	9	Cw*0302/4/5/6/8/9	529bp
11C	10	Cw*0302	206bp
12C	11	Cw*1202,*1301	449bp
13C	12	Cw*1202	597bp
14C	13	Cw*1203/6	453bp
15C	14	Cw*1402/3	541bp
16C	15	Cw*1502/3	373bp
17C	16	Cw*0102,*0404,*0604,*0707,*1302/3/4/5/6,*1701/2	500bp
18C	17	Cw*1601	512bp
19C	18	Cw*1602	512bp
20C	19	Cw*0602/3/4,*1204/5,*1602	324bp
21C	20	Cw*0202,*0602/3,*1204/5	501bp
22C	21	Cw*0704	563bp
23C	22	Cw*0707,*1701/2	475bp
PT 162	23	single 162bp=Cw*0602/4, 162&632bp=Cw*0801/3	
PT 93	24	Cw*0701/6/7	516bp
PT 94	25	Cw*0702/3/10	302bp
PT 95	26	Cw*0703, A*2604	494bp

FIGURE 14

Isoelectric focusing of HLA class 1 and β_2-microglobulin in colorectal cancer cell line HCA-7 and corresponding normal B cell line EVA-1224.



HCA-7 and EVA-1224 cells were metabolically labeled with [35S] methionine and immunoprecipitates isolated using antibody W6/32 as described in Materials and Methods, section 2.5.3. The immunoprecipitates were analyzed on a single dimensional isoelectric focussing gel.

EVA-1224 both express HLA-A2, HLA-B51 and –B37 and β_2-microglobulin, but HLA-A1 is not detectable in HCA-7.

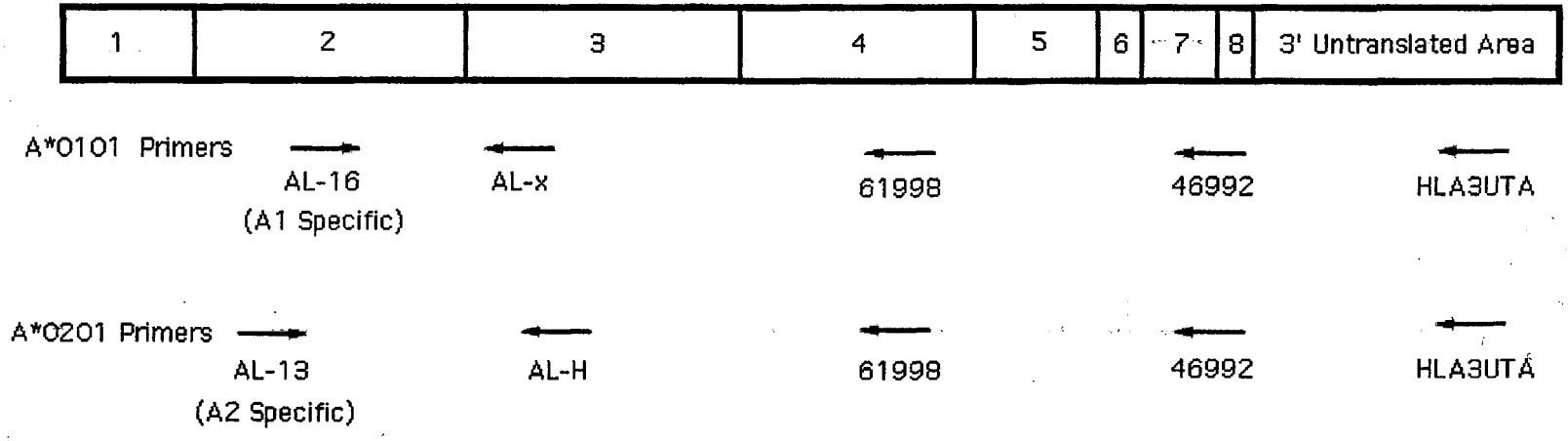
These data confirming lack of expression of the protein for HLA-A1 in HCA-7 and good evidence that it contained the gene, lead to an investigation at the mRNA level for HLA-A1 message. Preparations of mRNA were isolated from both HCA-7 and EVA-1224 cell lines according to the protocol described in Materials and Methods, section 2.4.1. RT-PCR was then performed, initially, using Random Hexamer Primers in the Superscript Reverse Transcriptase First strand synthesis system, followed by PCR using allele specific primers for HLA-A*0101 (and HLA-A*0201 for control purposes) as described in Materials and Methods, section 2.4.5. Figure 15 shows the different primer combinations used for mRNA analysis and their relative positions along the mRNA sequence. This approach revealed that HCA-7 contained only a short length of message for HLA-A*0101, compared to probable full-length message seen in EVA-1224 (see Figure 16). The data imply that the message for HLA-A*0101 in HCA-7 is intact from the beginning of exon 1 to at least the first half of exon 3, but 3′ from here is missing. Both the cell lines, however, appear to have full-length messages for HLA-A*0201.

In the first 12 bases of HLA-A*0101 exon 4 the sequence contains a run of 7 repeated cytidine (C) residues: 5′---GACCCCCCCAAG---3′. In certain rare individuals this region has been shown to include an additional cyditine, increasing the run of cytidines to eight (Bunce et al., 1999). Individuals with this frame shift of an additional cytidine have been reported not to express the gene product and the allele concerned has been designated "null". Primers designed to analyze this region by ARMS-PCR were therefore used to examine the beginning of exon 4 in HLA-A*0101 in the cell lines HCA-7 and EVA-1224. Assay conditions are described in Materials and Methods, section 2.4.2. The preliminary results indicated that EVA-1224 typed as having an HLA-A*0101 allele and HCA-7 an HLA-A*0101"null" allele, as shown in Figure 17.

Sequencing of this cytidine repeat region was performed by PCR using HLA-A locus specific primers (ATGTGTGTGGGGGTCTGAG [intron 3, bp 362-380]

FIGURE 15

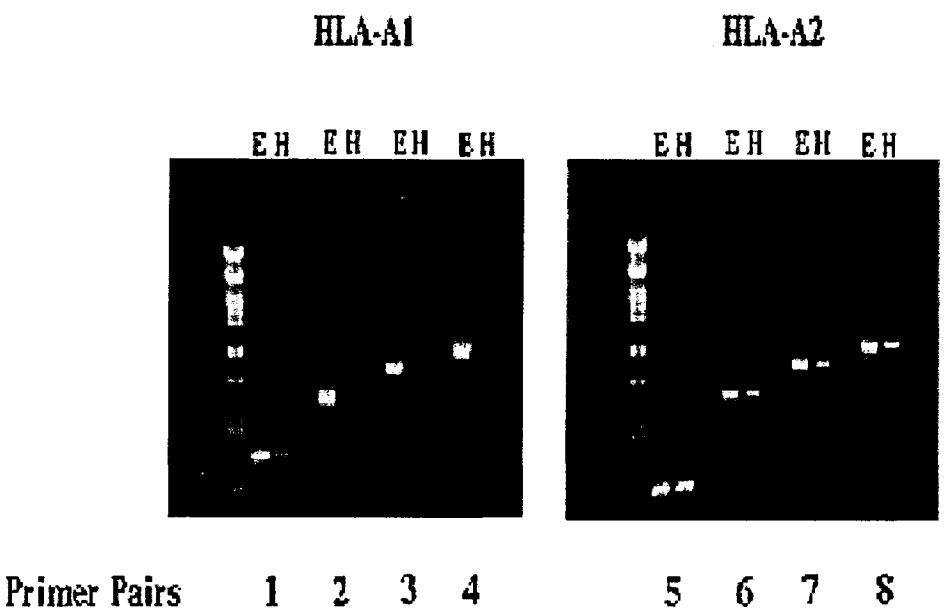
Diagram of HLA class I mRNA showing exon boundaries and position of primers used for ARMS RT-PCR analysis of HLA-A*0101 and HLA-A*0201.



The ARMS-PCR primers shown were used in a RT-PCR reaction in the conditions described in Materials and Methods, section 2.4.5. The numbers refer to exons (1-8) with a 3′ untranslated region indicated.

FIGURE 16

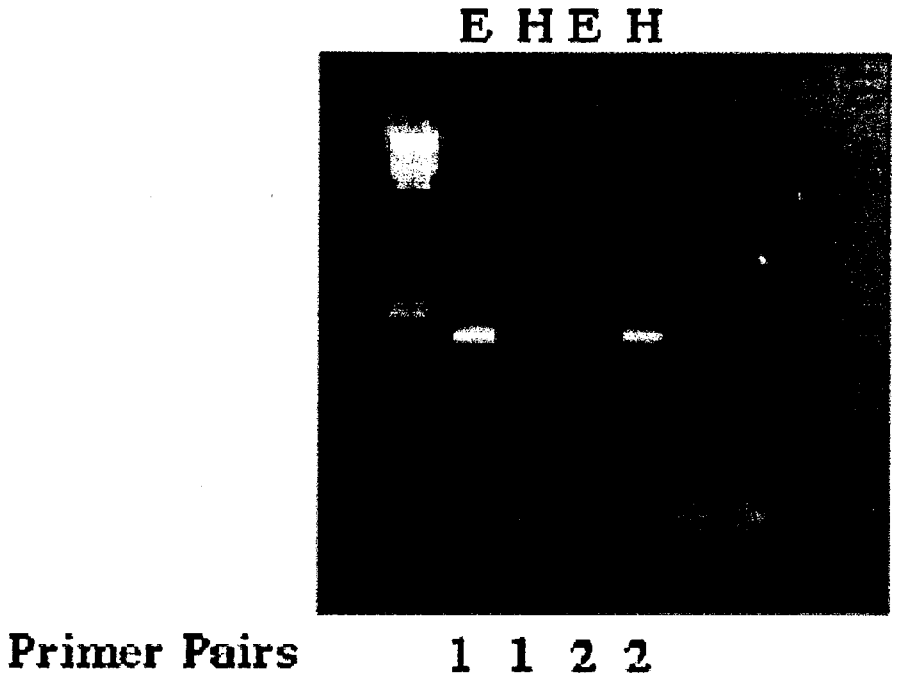
RT-PCR analysis of HLA-A*0101 and HLA-A*0201 in the colorectal cancer cell line HCA-7 and corresponding normal B cell line EVA-1224.



mRNA was isolated from the cell lines and RT-PCR performed with sequence specific primers, as shown in Figure 15, using the conditions described in Materials and Methods, section 2.4.5. Primer pairs used were: 1 AL-16 and AL-x; 2, AL-16 and 61998; 3, AL-16 and 46992; 4, AL-16 and HLA3UTA, for HLA-A*0101 and; 5, AL-13 and AL-H; 6, AL-13 and 61998; 7, AL-13 and 46992; 8, AL-13 and HLA3UTA, for HLA-A*0201. Samples were: E - EVA-1224 and H- HCA-7.

FIGURE 17

ARMS-PCR of cytidine repeat region in exon 4 of HLA-A*0101 in the colorectal cancer cell line, HCA-7 and in the corresponding normal B cell line, EVA-1224..



The cytidine repeat region in HCA-7 and EVA-1224 was analyzed by ARMS-PCR using the primers and conditions described in Materials and Methods, section 2.4.2. Primer combination 1 is specific for 7 cytidine repeats and primer combination 2 is specific for 8 cytidine repeats. Samples were: E - EVA-1224 and H- HCA-7.

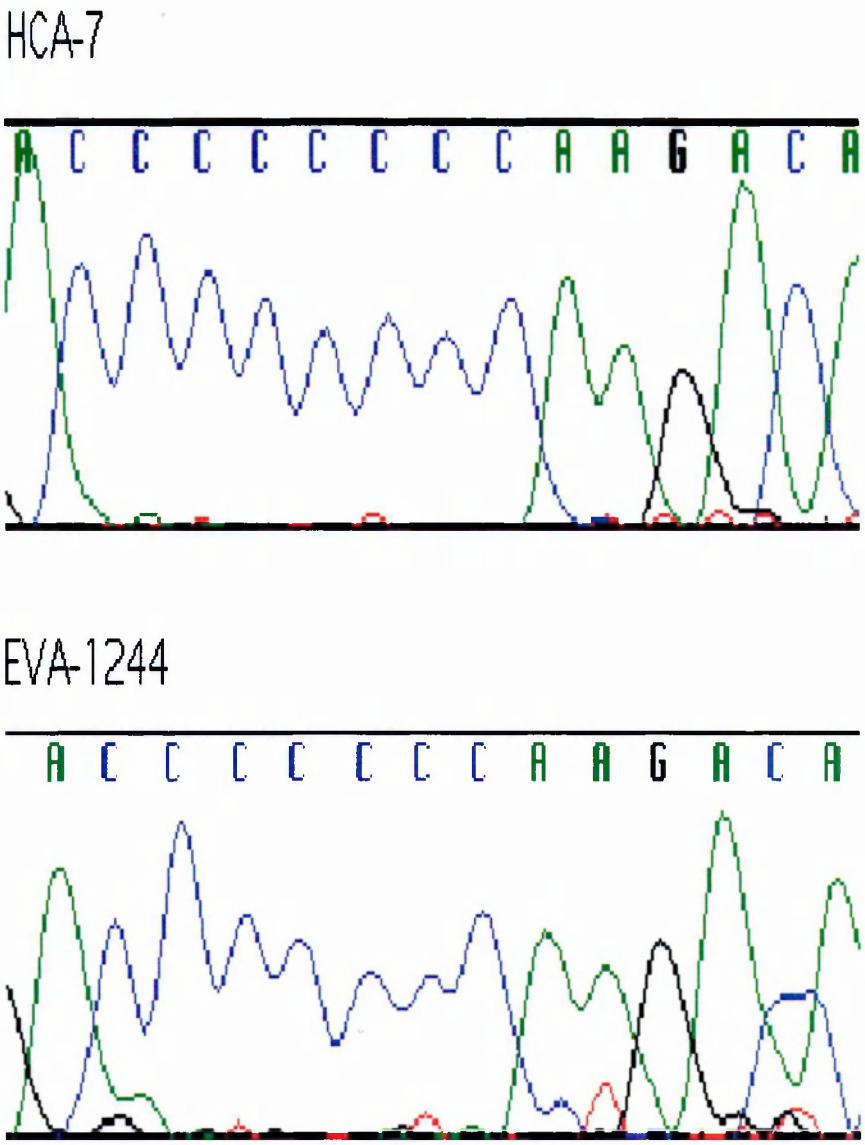
TABLE 11

Sequencing of clones from PCR products of the colorectal cancer cell line HCA-7 and lymphoblastoid cell line EVA-1224, which are derived from the same individual.

Cell line and Clone number		Sequence	Allele
HCA-7	1	CAGACCCCCCCCAAGA	A1"Null"
	2	CAGACCCCCCCCAAGA	A1"Null"
	3	CAGACGCCCCCAAAA	A2
	4	CAGACGCCCCCAAAA	A2
	5	CAGACCCCCCCCAAGA	A1"Null"
	6	CAGACGCCCCCAAAA	A2
	7	CAGACCCCCCCCAAGA	A1"Null"
	8	CAGACCCCCCCCAAGA	A1"Null"
EVA 1224	1	CAGACGCCCCCAAAA	A2
	2	CAGACGCCCCCAAAA	A2
	3	CAGACGCCCCCAAAA	A2
	4	CAGACCCCCCCCAAGA	A1
	5	CAGACGCCCCCAAAA	A2
	6	CAGACCCCCCCCAAGA	A1
	7	CAGACGCCCCCAAAA	A2
	8	CAGACCCCCCCCAAGA	A1

FIGURE 18

Sequence data from the cytidine repeat region of HLA-A*0101 of representative clones from HAC-7 and EVA-1224.



HLA-A* locus ARMS-PCR products from HAC-7 and EVA-1224 were cloned in the PCR-Script AMP cloning system and resulting clones sequenced using the Big-Dye protocol and analysed on an ABI 377 sequencer. (See Materials and Methods, sections, 2.4.2, 2.4.9 and 2.4.10.) Representative sequence traces for HLA-A*0101 are shown for HCA-7 upper panel and EVA-1224, lower panel.

forward primer and CTGCCATGTGCAGCATGAG [exon4, bp 846-865 reverse primer]) in the conditions detailed in Materials and Methods, section 2.4.2. This was followed by cloning in PCR-Script™ Amp Cloning System and sequencing using the Big-Dye automated sequencing technique as described in Materials and Methods, sections 2.4.9 and 2.4.10 respectively. Eight clones were sequenced from both EVA-1224 and HCA-7 and the results are shown in Table 11. Three of the clones from EVA-1224 sequenced as HLA-A*0101, containing 7 repeated cytidines, and the other five as HLA-A*0201. In HCA-7, three of the eight clones sequenced as HLA-A*0201 whilst the remaining five clones showed a sequence containing 8 repeated cytidines (see Figure 18) which is similar to the HLA-A*0101"null" allele.

3.4. Re-expression, using an inducible expression system, of HLA class I and β_2-microglobulin.

The plasmids (pcDNA-β_2M), containing an insert of β_2-microglobulin including leader peptide sequence, and (pcDNA-β_2M-15A2), containing leader peptide sequence, β_2-microglobulin, a 15 amino acid linker and HLA-A*0201, have been reported earlier (Toshitani et al., 1996). The restriction sites of these constructs were changed to 5' *Nhe I* and 3' *Hind III* as described in Materials and Methods, section 2.4.11, followed by ligation into the vector (pIND). These plasmids were named (pIND-β_2M) and (pIND-β_2M-15A2) respectively. Mini preparations were grown up and used to confirm the sequence of each insert. The sequences are shown in Figure 19 (pIND-β_2M) and Figure 20 (pIND-β_2M-15A2). Following confirmation of the sequences the plasmids (pIND-β_2M) and (pIND-β_2M-15A2) were grown up as maxi preparations in an endotoxin free system as described in Materials and Methods section 2.4.12.

Confirmation that the plasmid (pIND-β_2M-15A2) contained an insert that encoded for a protein of the expected size, was achieved by performing an *in vitro*-translation assay. The protocol used for the TnT coupled Reticulocyte Lysate system is described in section 2.4.13 of Materials and Methods. After

FIGURE 19

Sequence of the β_2-microglobulin construct (pIND-β_2M) showing the position of PCR primers used to change the cloning sites, and the restriction sites used to insert into vector pIND.

Nhe I site

CGG**GCTAGC**AGCATGTCTCGCTCCGTGGCCTTAGCTGTGCTCGCGCTACTCTCTCTTTCTGGCCTGGAGGCTATCCAGCGTACTCCAAA

GATTCAGGTTTACTCACGTCATCCAGCAGAGAATGGAAAGTCAAATTTCCTGAATTGCTATGTGTCTGGGTTTCATCCATCCGACATTG

AAGTTGACTTACTGAAGAATGGAGAGAGAATTGAAAAAGTGGAGCATTCAGACTTGTCTTTCAGCAAGGACTGGTCTTTCTATCTCTTG

TACTACACTGAATTCACCCCCACTGAAAAAGATGAGTATGCCTGCCGTGTGAACCATGTGACTTTGTCACAGCCCAAGATAGTTAAGTG

GGATCGAGACATGGGATCGAGACATGTAA**AAGCTT**TCA

Hind III site

The 5′ primer is shown in red, indicating a Nhe I site used for insertion into the vector (pIND), and the 3′ primer is shown in blue, with a Hind III site highlighted in red.

FIGURE 20

Sequence of the β_2-microglobulin/HLA-A*0201 construct (pIND-β_2M15-A2) showing the positions of PCR primers used to change the cloning sites, and the restriction sites used to insert into vector pIND.

Nhe I site B_2-microglobulin

CGG**GCTAGC**AGCATGTCTCGCTCCGTGGCCTTAGCTGTGCTCGCGCTACTCTCTCTTTCTGGCCTGGAGGCTATCCAGCGTACTCCAAA
GATTCAGGTTTACTCACGTCATCCAGCAGAGAATGGAAAGTCAAATTTCCTGAATTGCTATGTGTCTGGGTTTCATCCATCCGACATTG
AAGTTGACTTACTGAAGAATGGAGAGAGAATTGAAAAAGTGGAGCATTCAGACTTGTCTTTCAGCAAGGACTGGTCTTTCTATCTCTTG
TACTACACTGAATTCACCCCCACTGAAAAAGATGAGTATGCCTGCCGTGTGAACCATGTGACTTTGTCACAGCCCAAGATAGTTAAGTG
GGATCGAGACATG.....

Linker

.....GAGGTGGCGGATCCGGCGGAGGTGGCTCGGGTGGCGGCGGATCC.....

HLA-A*0201

.....GGATCTCACTCCATGAGGTATTTCTTCACATCCGTGTCCCGGCCCGGCCGCGGGGAGCCCCGCTTCATCGCAGTGGGCTACGT
GGACGACACGCAGTTCGTGCGGTTCGACAGCGACGCCGCGAGCCAGAGGATGGAGCCGCGGGCGCCGTGGATAGAGCAGGAGGGTCCGG
AGTATTGGGACGGGGAGACACGGAAAGTGAAGGCCCACTCACAGACTCACCGAGTGGACCTGGGGACCCTGCGCGGCTACTACAACCAG
AGCGAGGCCGGTTCTCACACCGTCCAGAGGATGTATGGCTGCGACGTGGGGTCGGACTGGCGCTTCCTCCGCGGGTACCACCAGTACGC
CTACGACGGCAAGGATTACATCGCCCTGAAAGAGGACCYGCGCTCTTGGACCGCGGCGGACATGGCAGCTCAGACCACCAAGCACAAGT
GGGAGGCGGCCCATGTGGCGGAGCAGTTGAGAGCCTACCTGGAGGGCACGTGCGTGGAGTGGCTCCGCAGATACCTGGAGAACGGGAAG
GAGACGCTGCAGCGCACGGACGCCCCCAAAACGCATATGACTCACCACGCTGTCTCTGACCATGAAGCCACCCTGAGGTGCTGGGCCCT
GAGCTTCTACCCTGCGGAGATCACACTGACCTGGCAGCGGGATGGGGAGGACCAGACCCAGGACACGGAGCTCGTGGAGACCAGGCCTG
CAGGGGATGGAACCTTCCAGAAGTGGGCGGCTGTGGTGGTGCCTTCTGGACAGGAGCAGAGATACACCTGCCATGTGCAGCATGAGGGT
TTGCCCAAGCCCCTCACCCTGAGATGGGAGCCGTCCTCCCAGCCCACCATCCCCATCGTTGGGCATCATTGCTGGCCTGGTTCTCTTTG
GAGCTGTGATCACTGGAGCTGTGGTCGCTGCTGTGATGTGGAGGAGGAAGAGCTCAGATAGAAAAGGAGGGAGCTACTCTCAGGCTGCA
AGCAGTGACAGTGCCCAGGGCTCTGATGTGTCTCTCACAGCTTGTAAAGTGTGATGAGGATCCTCTAGAGTCGACCTC**AAGCTT**GGGC

Hind III site

The 5'primer is shown in red, indicating a Nhe I site used for insertion into the vector pIND, and the 3'primer is shown in blue, with a Hind II site in red.

separation of products by electrophoresis, autoradiography revealed a single band at approximately 57 kDa (see Fig 21 which shows results from duplicate experiments) and is the expected size, adding together 12 kDa and 45 kDa, for β_2-microglobulin and HLA-A*0201 respectively. A similar *in vitro*-translation assay was performed for the (pIND-β_2M) insert that confirmed a product of 12 kDa, data not shown.

3.4.1 Transfections into the colorectal cell line, DLD-1.

The colorectal cell line DLD-1 lacks expression of HLA-A, B and C due to two non-complementary heterozygous mutations in the β_2-microglobulin gene (see section 3.1.1 above) and therefore provides a good candidate cell line for studying the re-express HLA molecules at the cell surface. This was attempted using the ecdysone inducible expression system.

Prior to transfecting the colorectal cell line, DLD-1, a killing curve assay was performed to ascertain the concentration required for complete cell death when using the two selection agents Geneticin (G418) and Zeocin. Cultures of the cells were grown in increasing concentrations of the agents and cell viability counted after 10 days of exposure to the agents. From the data in Figures 22 and 23, concentrations of 1.5 mg/ml for G418 and 0.1mg/ml Zeocin were chosen. (The Figures also show the killing curves for the cell line CHO used in later experiments, see section 3.4.3).

The plasmid (pIND-β_2M) and Ecdysone receptor plasmid (pVgRXR) were co-transfected into the DLD-1 cell line and primary clones resistant to the selection agents, G418 and Zeocin, grown out as described in Materials and Methods section 2.4.14. These primary clones were picked and sub-cultured in 96 well plates, in duplicate. One culture was stimulated for 24 hours with Ponasterone A (1μM), whilst the second culture had no Ponasterone A added and served as an unstimulated control. These duplicate cultures were then assayed by ELISA using antibody L368 (anti-β_2-microglobulin) according to the protocol in

FIGURE 21

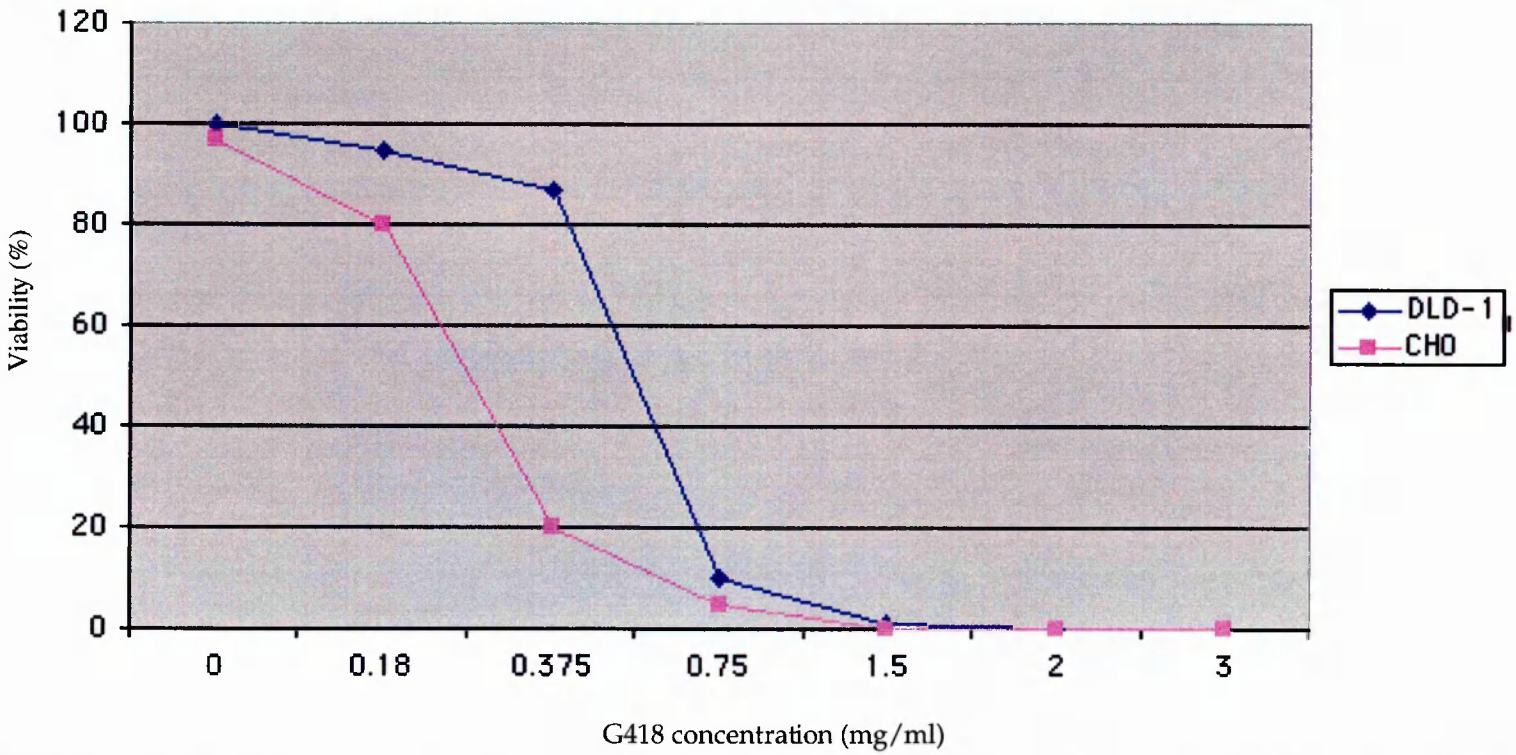
Autoradiograph from an *invitro* translation assay of the (pIND-β_2M15-A2) construct.

Track 1 Track2 Track 3

The invitro translation assay was peformed as described in Materials and Methods, section 2.4.13 using the TnT coupled reticulocyte lysate system. Following the assay the products were electrophoresed on a 12.5% polyacrylamide gel and labeled products detected by autoradiography.
The assay was performed in duplicate (Tracks 1 and 2) and molecular weight size markers run in Track 3.

FIGURE 22

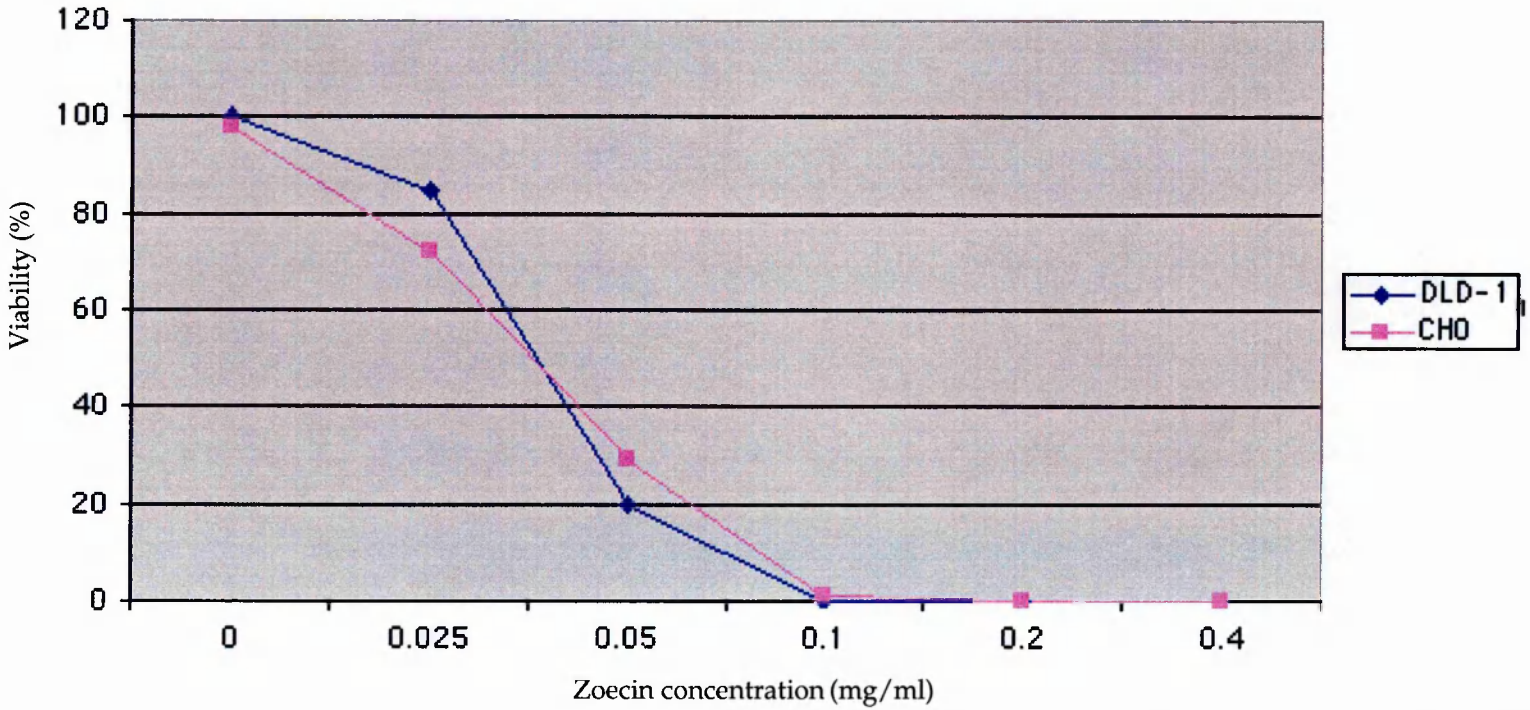
G418 killing curves for the cell lines DLD-1 and CHO.



The cell lines DLD-1 and CHO were grown in DMEM and Iscove's modified DMEM respectively (as described in Materials and Methods Section 2.1.1), with the addition of different concentrations of G418. Cultures were grown for 10 days then assessed for viability.

FIGURE 23

Zeocin killing curves for cell lines DLD-1 and CHO.



The cell lines DLD-1 and CHO were grown in DMEM and Iscove's modified DMEM respectively (as described in Materials and Methods section 2.1.1), with the addition of different concentrations of Zeocin. Cultures were grown for 10 days then assessed for viability.

Materials and Methods section 2.3.2. The data for DLD-1/(pIND-β_2M) primary clones are shown in Figure 24.

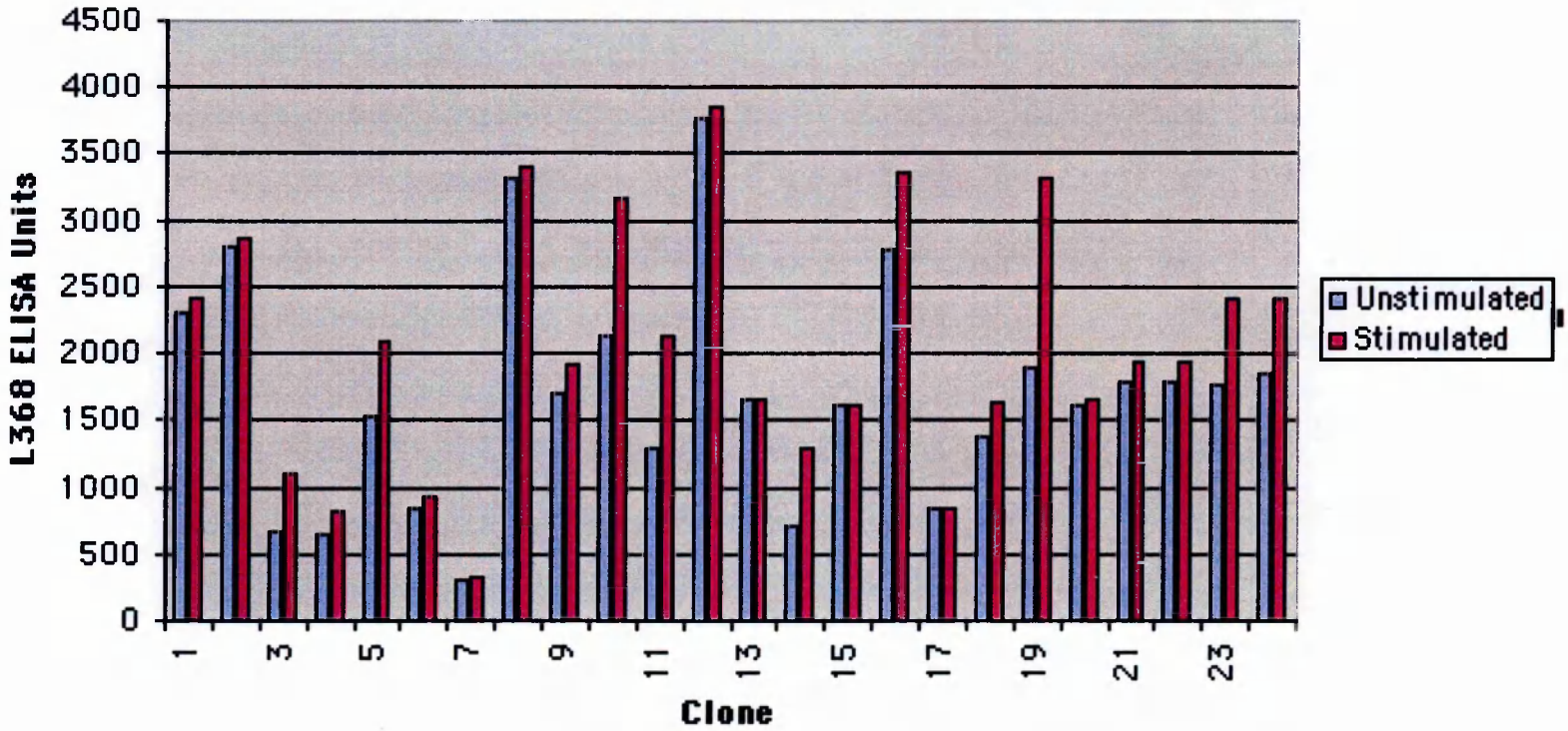
A similar co-transfection of (pVgRXR) and (pIND-β_2M-15A2), the plasmid containing the β_2-microglobulin-HLA-A*0201 construct, was performed and primary clones picked and assayed by ELISA as described above; data for these primary clones are shown in Fig 25.

From each of the transfections, two primary clones were chosen that grew well in selection medium and, from the ELISA data, contained some cells which reacted with the antibody L368 after stimulation and apparently expressed either β_2-microglobulin or the β_2-microglobulin-HLA-A*0201 construct. These were clones 3 and 19 for DLD-1-(pIND-β_2M) and 18 and 24 for DLD-1-(pIND-β_2M-15A2) (see Figures 24 and 25). In an attempt to speed up the isolation of cells reactive with the antibody L368, clones were grown up and stimulated with Ponasterone A to express the constructs. Positive cells were then selected by staining the cells with antibody L368 followed by selection using magnetic beads coated with goat-anti mouse, as described in Materials and Methods section 2.4.15. Seven days after each magnetic bead sort the number of positive cells in the culture was estimated by immunofluorescence. Four rounds (of growing, stimulating and sorting) were performed for each clone and, as shown in Table 12, the number of positive cells appeared to increase with each additional sort.

On completion of four sorts with magnetic beads, the positively selected cells were grown for approximately 28 days, 4 passages, and then samples analyzed by ELISA assay. This was to check the responsiveness of the cells to Ponasterone A for construct expression. Similar treatment was performed with the negative, magnetic bead unselected, portion of the fourth sort. As can be seen from Table 13 the two clones 18 and 24 from DLD-1-(pIND-β_2M-15A2) were not responsive to Ponasterone A. The positives from the 4^{th} sort of both these clones reacted with antibody L368 when they had not been stimulated with Ponasterone A. Similar results were seen after 7 passages (see Table 13). The negatives from the 4^{th} sort appeared not to react with L368. Magnetic beads did not efficiently sort clones

FIGURE 24

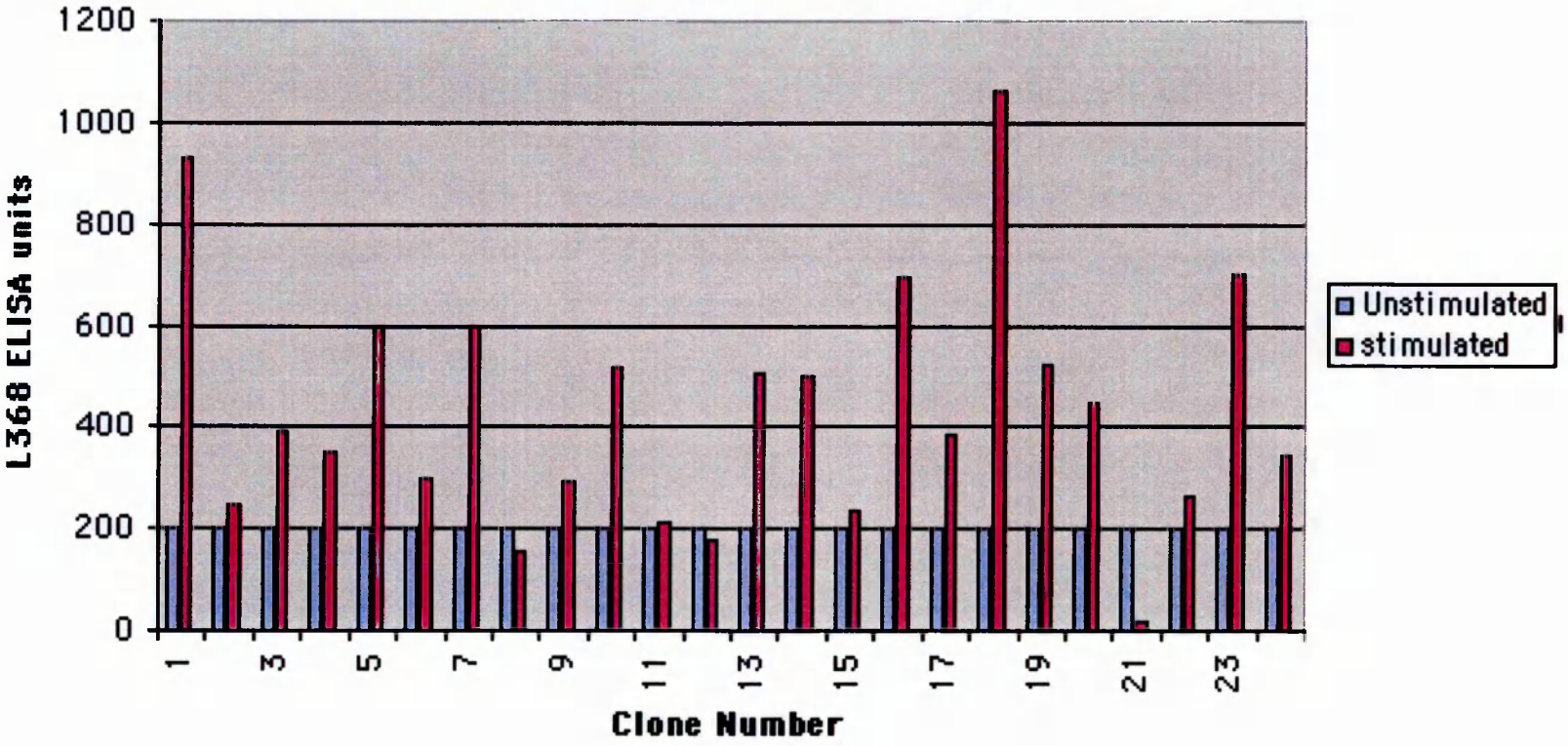
ELISA analysis of primary clones of DLD-1 (pIND-β2M).



Colorectal cell line DLD-1 was co-transfected with (pIND-β_2M) and (pVgRXR) and clones selected in G418 and Zeocin as described in Materials and Methods, section 2.4.14. Primary clones were stimulated with Ponasterone A (each clone with a duplicate unstimulated control) and analysed by ELISA, using antibody L368, as described in Materials and Methods, section 2.3.2.

FIGURE 25

ELISA analysis of primary clones of DLD-1 (pIND-β2M15-A2).



Colorectal cell line DLD-1 was co-transfected with (pIND- β2M15-A2) and (pVgRXR) and clones selected in G418 and Zeocin as described in Materials and Methods, section 2.4.14. Primary clones were stimulated with Ponasterone A (each clone with a duplicate Unstimulated control) and analysed by ELISA, using antibody L368, as described in Materials and Methods, section 2.3.2.

TABLE 12

Analysis, by immunofluorescence, of DLD-1 transfected clones following stimulation for 24 hours with Ponasterone A (1mM) and sorting with magnetic beads* .

Cell line (Construct) Clone Number	Pre-sort	After 1st sort**	After 4th sort**
DLD-1-(pIND-β_2M)			
Clone 3	>1%	10%	90%
Clone 19	>1%	15%	ND
DLD-1-(pIND-β_2M-15A2)			
Clone 18	>1%	10%	95%+
Clone 24	>1%	ND	50%+

* Clones were stained with L368 antibody and selected with Goat anti-mouse coated magnetic beads as described in Materials and Methods, section 2.4.15. One week later clones were analysed by immunofluorescence using antibody L368, followed by Goat anti-mouse FITC.

** Figures shown represent the number of cells positively staining (%)

+ Analysis performed using antibody W6/32.

TABLE 13

Analysis by ELISA,* of clones of transfected DLD-1 cells after four sorts with magnetic beads.

Construct-Clone	After 4 passages		After 7 passages	
	UNSTIM	STIM^{+}	UNSTIM	STIM^{+}
DLD-1- (pIND-β_2M15-A2)				
Clone 18 (Positives from sort)	3813	3797	4000	4000
Clone 18 (Negatives from sort)	414	431	495	463
Clone 24 (Positives from sort)	4000	4000	4000	4000
Clone 24 (Negatives from sort)	643	816	816	815
DLD-1- (pIND- β_2M)				
Clone 3 (Positives from sort)	2662	2777	4000	4000
Clone 3 (Negatives from sort)	2252	2083	3444	3144
Clone 19 (Positives from sort)	4000	4000	4000	4000
Clone 19 (Negatives from sort)	4000	4000	4000	4000

* ELISA using antibody L368 as described in Materials and Methods, section 2.3.2. Results are shown as ELISA units (Maximum reading 4000)

+ Stimulated with Ponasterone A as described in Materials and Methods and analysed 24 hours after stimulation.

from the DLD-1-(pIND-β_2M) transfection as the data show little difference in the ELISA results for the positive and negative sorts (see Table 13).

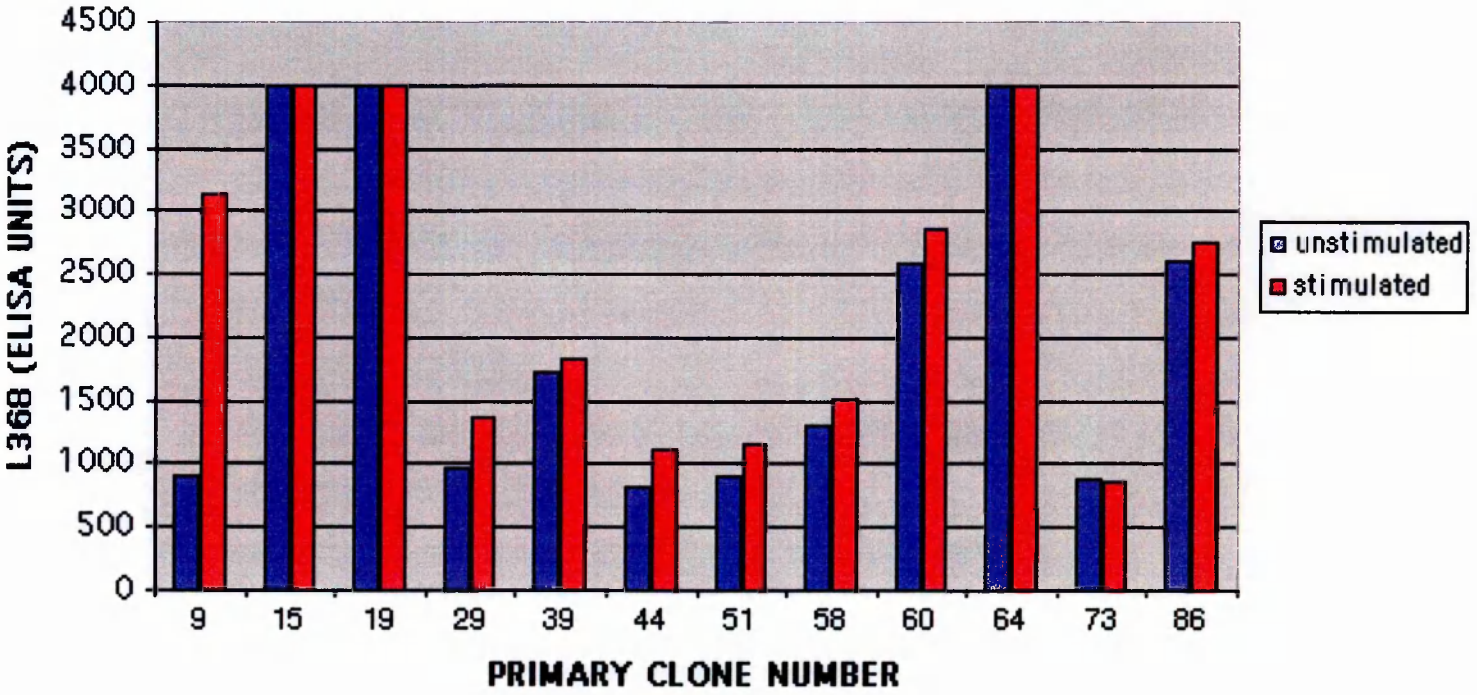
3.4.2 Transfections into the cell line CHO.

The transfection experiments using the plasmids (pIND-β_2M) and (pIND-β_2M-15A2) in an inducible expression system with the colorectal cell line DLD-1 failed to produce a stable clone that re-expressed HLA in response to the inducer Ponasterone A (see section 3.4.2 above). In an attempt to test the inducible expression system in a cell line that was easier to grow and clone, a series of transfections was performed in the Chinese hamster ovarian cell line, CHO. To check that CHO would make a good candidate cell line to work with, it was assayed a) by ELISA with the antibody L368 (anti-β_2-microglobulin) and b) by immunofluorescence with W6/32 (anti-HLA-A,-B&-C/β_2-microglobulin) and BB7.2 (anti-HLA-A2). The CHO cells were negative in both assays with all antibodies. In addition, the concentration of selection agents G418 and Zeocin, required to kill all CHO cells in 10 days, was determined and found to be 1mg/ml and 0.1mg/ml, respectively (see section 3.4.2 and Figures 22 and 23).

Either (pIND-β_2M) or (pIND-β_2M-15A2) were co-transfected with the Ecdysone receptor plasmid (pVgRXR) into CHO cells and primary clones resistant to the selection agents, G418 and Zeocin, grown out as described in Materials and Methods section 2.4.14. Primary clones were picked and sub-cultured in duplicate, in 96 well plates. One culture was stimulated for 24 hours with Ponasterone A (1μM), whilst the second culture had no Ponasterone A added and served as an unstimulated control. These duplicate cultures were then assayed by ELISA using antibody L368 (anti-β_2-microglobulin) as described in Materials and Methods section 2.3.2. Approximately 100 clones were assayed for each transfection and the clones with results above background are show in Figure 26 for (pIND-β_2M-15A2) and Figure 27 for (pIND-β_2M).

FIGURE 26

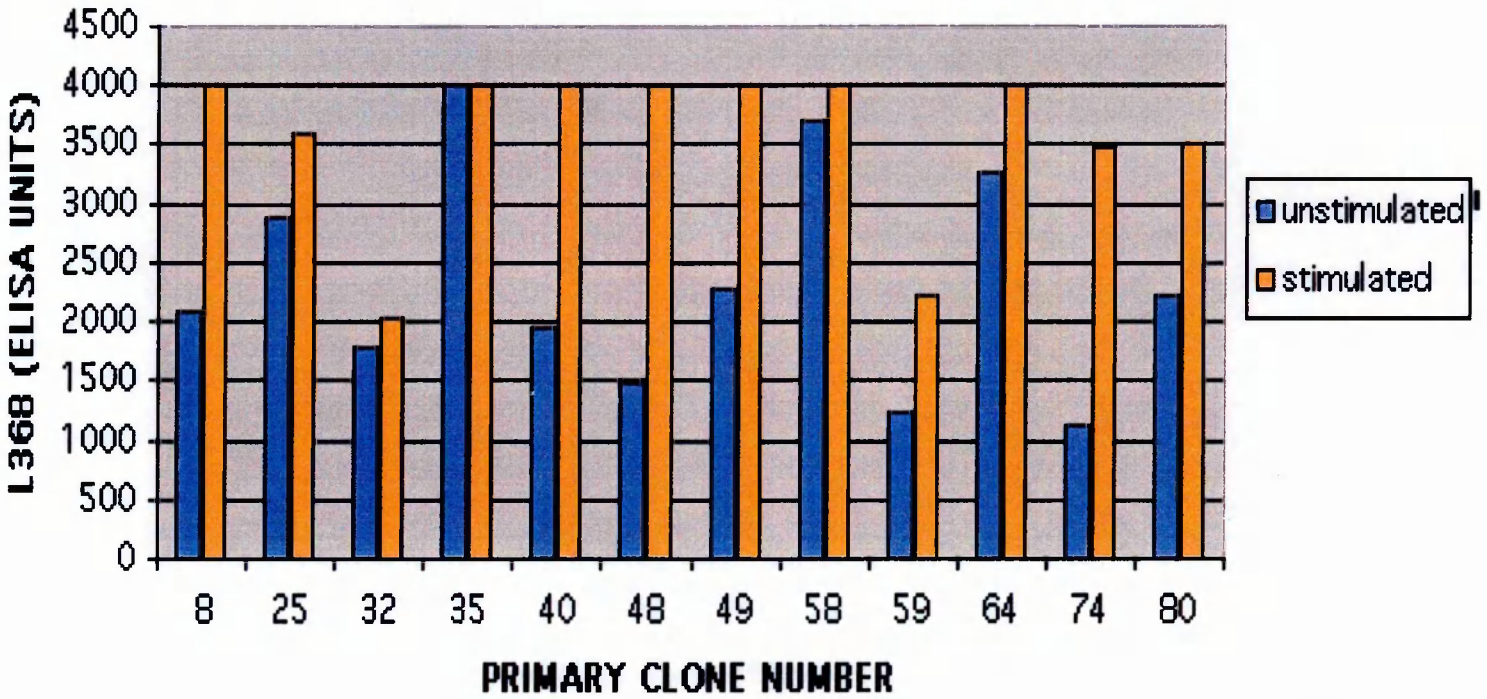
ELISA analysis of primary clones of CHO (pIND-β_2M15-A2).



The CHO cell line was co-transfected with (pIND-β_2M15-A2) and (pVgRXR) and clones selected in G418 and Zeocin as described in Materials and Methods, section 2.4.14. Primary clones were stimulated with Ponasterone A (each clone with a duplicate unstimulated control) and analysed by ELISA, using antibody L368, as described in Materials and Methods, section 2.3.3.

FIGURE 27

ELISA analysis of primary clones of CHO (pIND-β_2M).



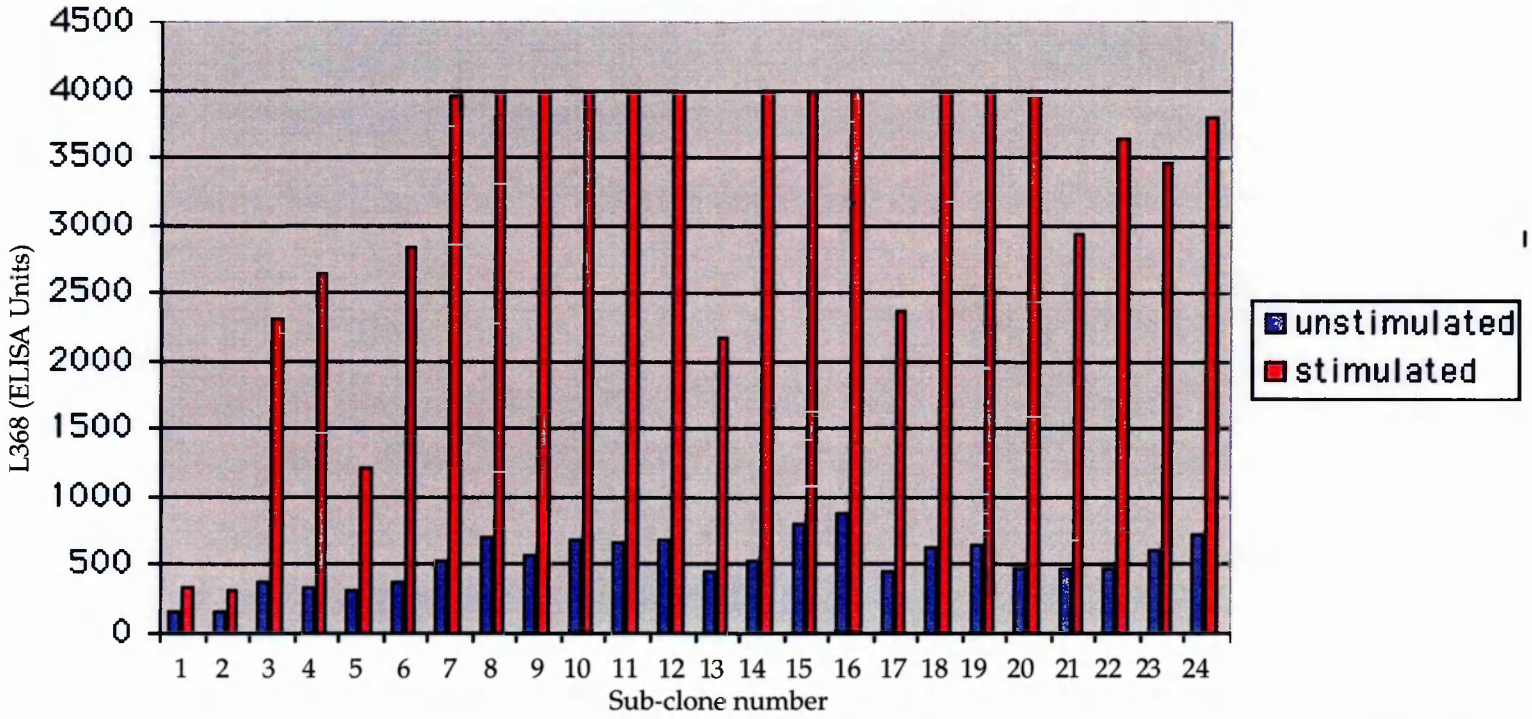
The CHO cell line was co-transfected with (pIND-β_2M) and (pVgRXR) and clones selected in G418 and Zeocin as described in Materials and Methods, section 2.4.14. Primary clones were stimulated with Ponasterone A (each clone with a duplicate unstimulated control) and analysed by ELISA, using antibody L368, as described in Materials and Methods, section 2.3.3.

One primary clone from each transfection was chosen to sub-clone, by limiting dilution, as described in Materials and Method section 2.4.16. The clones selected were those with the highest difference, from the ELISA data, between stimulated and background (unstimulated) duplicates. Primary clone 9 was chosen from the (pIND-β_2M-15A2) transfection and primary clone 48 from (pIND-β_2M). In the latter case there were several clones which demonstrated maximal readings for the ELISA assay (4000), but clone 48 had the lowest background (unstimulated) reading, see Figure 27. When the sub-clones had grown sufficiently to assay, they were set up in duplicate cultures, one being stimulated with Ponasterone A, and assayed by ELISA with antibody L368 as described above for the primary clones, and the other duplicate frozen for storage. Approximately 24 sub-clones were screened in each case and the results for (pIND-β_2M-15A2) clone 9 are shown in Figure 28 and for (pIND-β_2M) clone 48 in Figure 29. The best sub-clones were chosen by the same criterion used to select the primary clones. Thus, sub-clones 9/9, 9/11 and 9/20 were selected from the (pIND-β_2M-15A2) transfection and sub-clones 48/3 and 48/18 from the (pIND-β_2M) transfection.

The ELISA data indicated that the sub-clones could be induced to express a construct product that reacted with antibody L368, but these data did not differentiate between surface membrane and cytoplasmic expression. To address this, the sub-clones were initially induced with Ponasterone A for 24 hours, then stained by immunofluorescence with antibody L368 (details in Materials and Methods, section 2.3.1). Sub-clone 9/20 showed strong positive reactivity on 95% of the cells, whilst sub-clones 9/9, 9/11, 48/3 and 48/18 were only very weakly positive on 5-25% of the cells. In addition, the sub-clones from (pIND-β_2M-15A2) clone 9 were similarly examined with antibody BB7.2 (anti-HLA-A2) and again sub-clone 9/20 was strongly positive on 95% of cells and 9/9 and 9/11 were only faintly reactive on 10-25% of cells. These immunofluorescence experiments were repeated and analyzed by Flow cytometry, the FACScan data being shown in Figure 30 for sub-clones of (pIND-β_2M-15A2) clone 9 and Figure 31 for the two sub-clones of (pIND-β_2M) clone 48. These results indicate that the only clone inducibly expressing, at the cell surface, was (pIND-β_2M-15A2) sub-clone 9/20

FIGURE 28

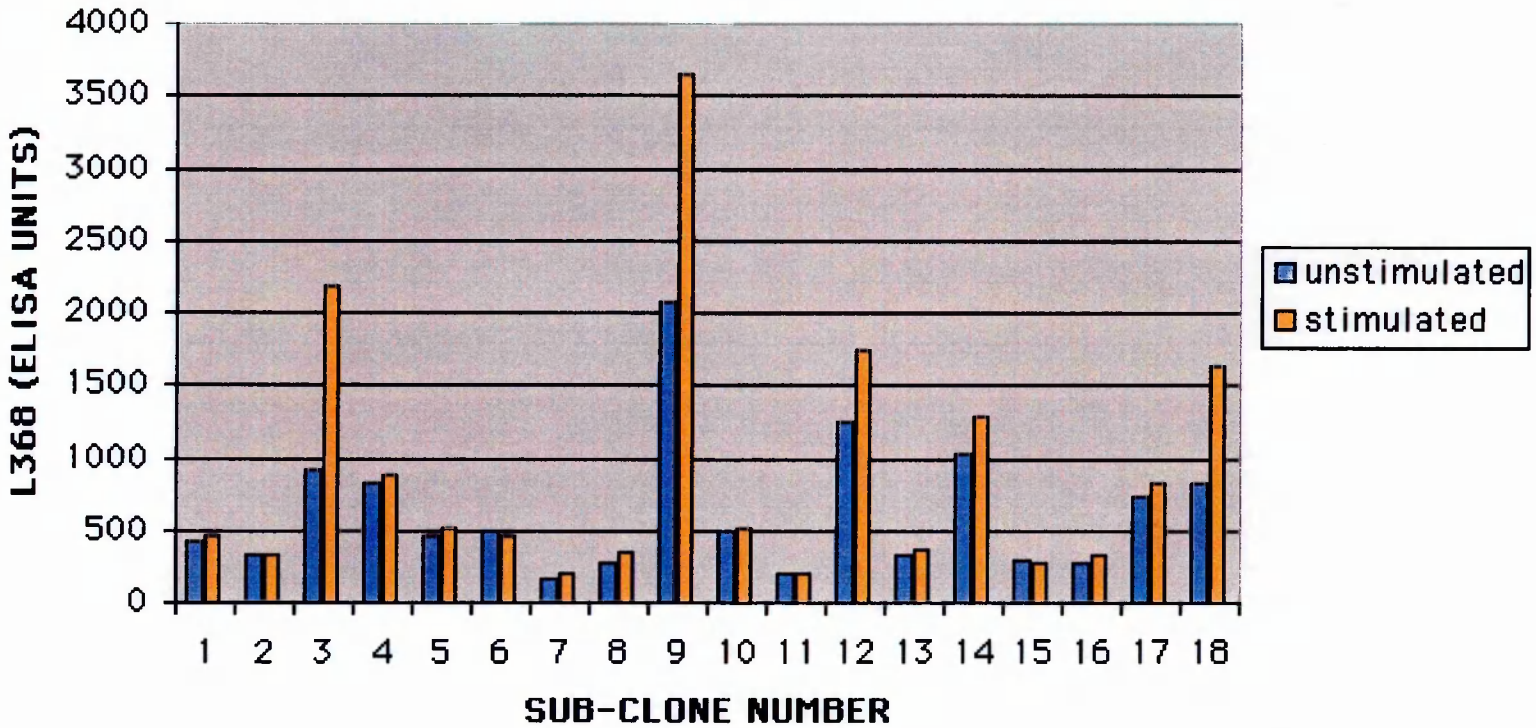
ELISA analysis of sub-clones of CHO (pIND-β_2M15-A2)-clone 9, after cloning by limiting dilution.



Clone 9 from the primary clones screened of the CHO (pIND-β_2M15-A2) transfection was sub-cloned by limiting dilution as Described in Materials and Methods, section 2.4.16. Sub-clones were stimulated with Ponasterone A (each sub-clone with a duplicate unstimulated control) and analysed by ELISA, using antibody L368, as described in Materials and Methods, section 2.3.3.

FIGURE 29

ELISA analysis of sub-clones of CHO (pIND-β_2M)-clone 48, after cloning by limiting dilution.



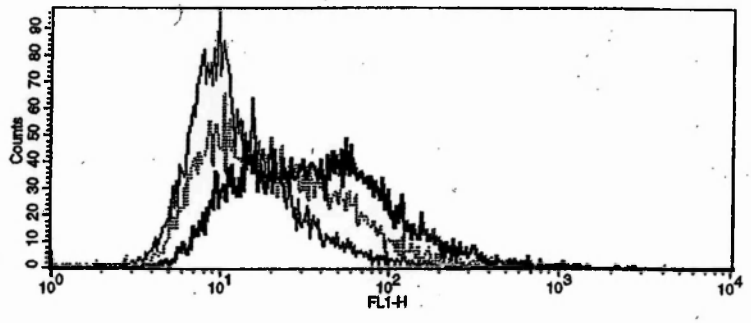
Clone 48 from the primary clones screened of the CHO (pIND-β_2M) transfection was sub-cloned by limiting dilution as described in Materials and Methods, section 2.4.16. Sub-clones were stimulated with Ponasterone A (each sub-clone with a duplicate unstimulated control) and analysed by ELISA, using antibody L368, as described in Materials and Methods, section 2.3.3.

FIGURE 30

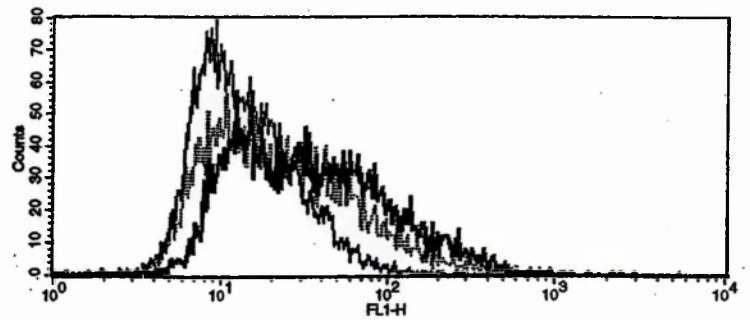
FACS ANALYSIS OF SUB-CLONES OF (pIND-β_2M15-A2) USING ANTIBODIES L368 (anti-β_2M) AND BB7.2 (anti HLA-A*2).

Subclones were stimulated with Ponasterone A and stained with antibody as described in Materials and Methods. Control (———) L368 (▬▬) BB7.2 (- - - - - - - -).

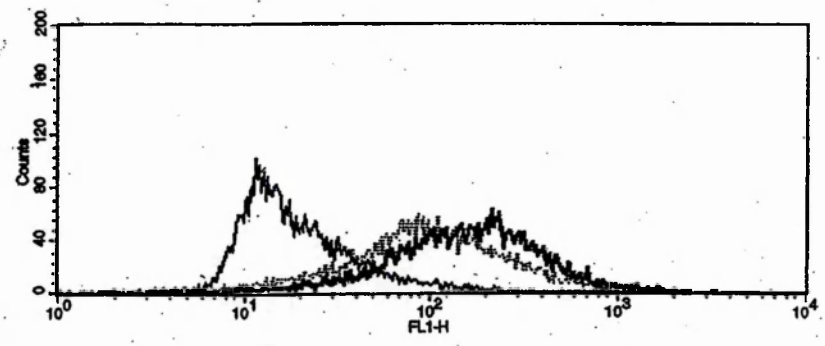
Sub-clone 9/9



Sub-clone 9/11



Sub-clone 9/20



Control Unstimulated

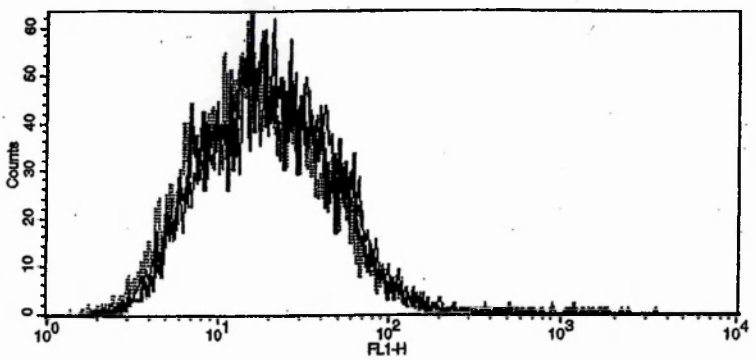
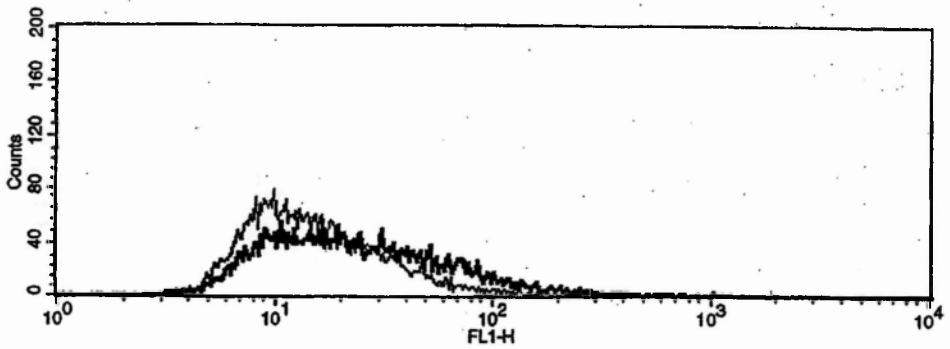


FIGURE 31

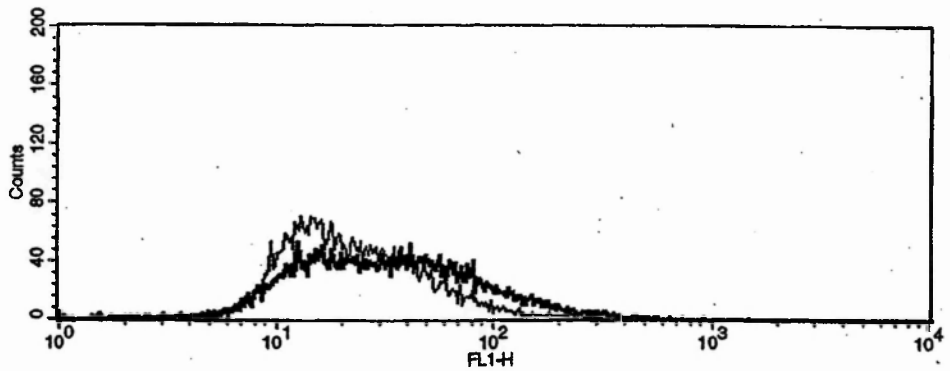
FACS ANALYSIS OF SUB-CLONES OF (pIND-β_2M) USING ANTIBODy L368 (anti-β_2M).

Subclones were stimulated with Ponasterone A and stained with antibody as described in Materials and Methods. Control (———) L368 (▬▬▬).

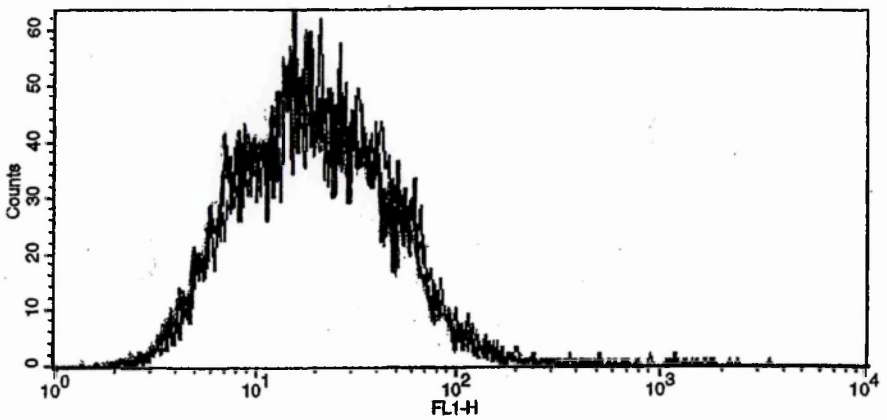
Sub-clone 48/3



Sub-clone 48/18



Control Unstimulated



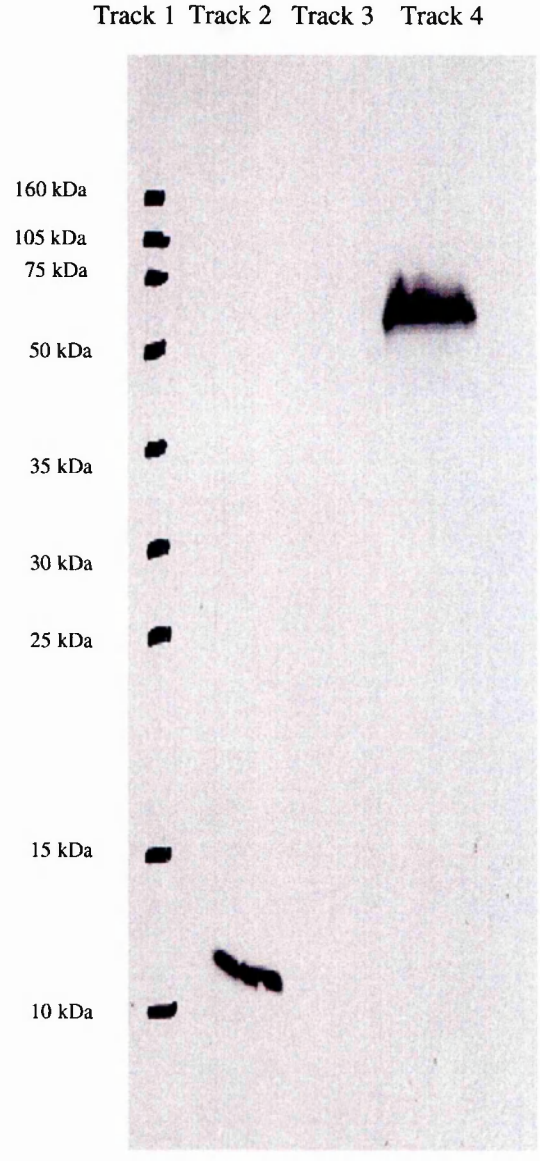
which, with reactivity seen with both antibodies L368 and BB7.2, was most likely expressing the construct (β_2-microglobulin-HLA-A*0201).

Confirmation that (pIND-β_2M-15A2) sub-clone 9/20 was expressing the (β_2-microglobulin-HLA-A*0201) construct was achieved by Western Blotting. Duplicate cultures of sub-clone 9/20 were established, then one culture stimulated with Ponasterone A for 24 hours, while the other culture contained medium only and served as an unstimulated control. Then the cultures were harvested, counted and lysed. Samples of lysates, with equivalent cell numbers, were then electrophoresed (in reducing conditions) by SDS-PAGE, following by overnight transfer and probing with antibody L368 as described in Materials and Methods section 2.4.2. The autoradiograph shown in Figure 32 detects a band at approximately 57 kDa in the stimulated sub-clone 9/20 track, which is absent in the unstimulated track. This band is the same size as the product seen in the *in vitro* translation experiment run with the plasmid (pIND-β_2M-15A2) prior to transfection (see Figure 21). The normal EBV transformed B cell line, Bristol-8, was included in the Western Blot (Figure 32) for control purposes, and exhibits a band at approximately 12 kDa, which is the expected size for β_2-microglobulin when run in reducing conditions in SDS-PAGE.

Expression of the (β_2-microglobulin-HLA-A*0201) construct by sub-clone 9/20 was investigated with respect to the concentration and time scale of stimulation with the inducer Ponasterone A. Concentrations of Ponasterone A, ranging from 0.001μM to 10μM were added to a series of duplicate cultures of sub-clone 9/20. After 25 hours the clones were assayed by ELISA, using the antibody L368 as described in Materials and Methods, section 2.3.2. The results, shown in Figure 33, reveal that at very low concentrations, between 0.001μM and 0.01μM very little induction is seen after 25 hours, but at concentrations above 1μM, maximum readings are seen in the ELISA assay. A similar series of duplicate sub-clone 9/20 cultures, with concentrations of Ponasterone A ranging from 0.25μM to 1μM, was monitored at 0, 1, 2, 4, 8, 18, 24 and 30 hours after adding the inducer. Stimulation was again measured by ELISA using antibody L368. Detectable reactivity with L368 was seen after two hours, and by approximately 18 hours the lowest concentration of Ponasterone A used

FIGURE 32

Western Blot of CHO sub-clone 9/20 cells for inducible expression of the (pIND-β_2M15-A2) construct when stimulated with Ponasterone A.



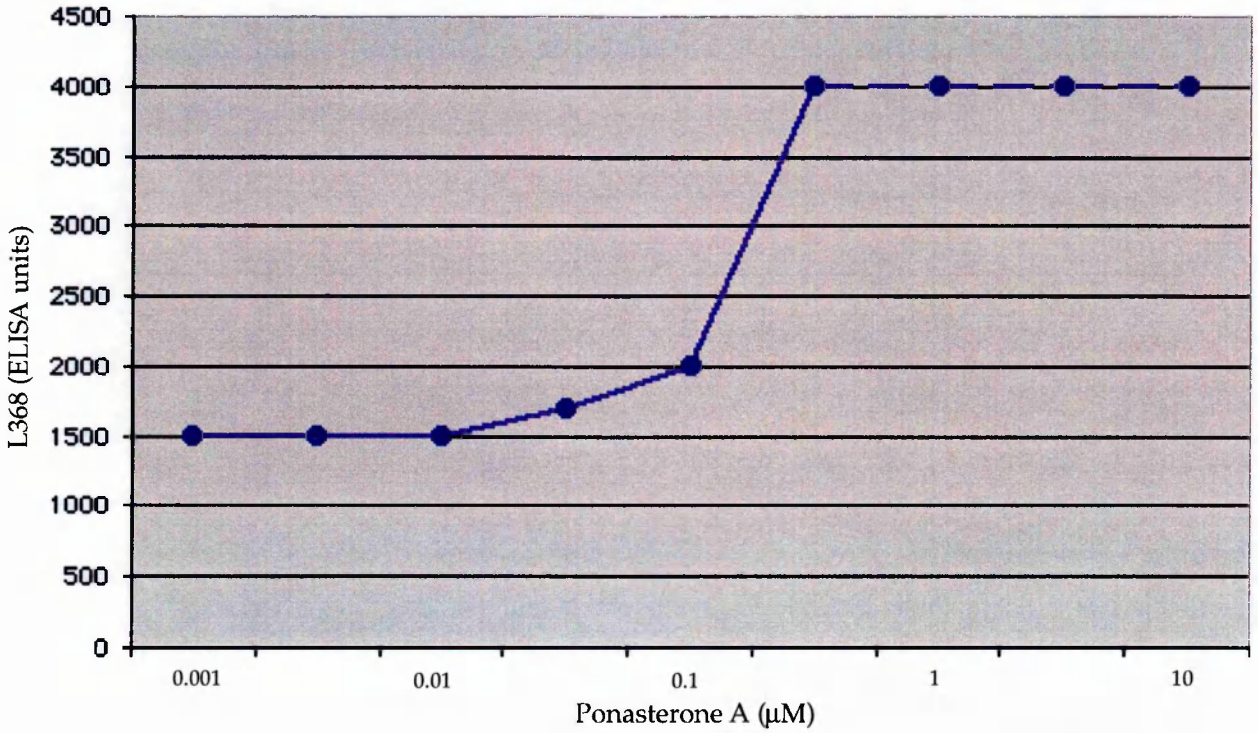
Cells were counted and lysed in RIPA buffer as described in Materials and Methods,section2.5.1. for Western Blotting, 1 x 10^6 cell equivalents were run in each track on an 18.5% polyacrylamide SDS gel and transferred and probed with antibody L368 as outlined in Materials and Methods section 2.5.2. Detection was by ECL.
Samples were: Track 1, Molecular weight markers; Track 2 Bristol-8 normal B cell line, used as control for endogenous $\beta_2$microglobulin; Track 3, CHO (pIND-β_2m15-A2) sub-clone 9, unstimulated control; Track 4 CHO (pIND-β_2m15-A2) sub-clone 9 stimulated for 24 hours with Ponasterone A (1µm) before harvesting the cells.

(0.25μM) had induced a maximum response in the ELISA (see Figure 34). Inducible expression on the cell surface was also analyzed with respect to time and concentration of inducer. A similar series of experiments to those just described, was performed using different times and concentrations of Ponasterone A, and the cells then examined by immunofluorescence using antibody W6/32, followed by Flow cytometric analysis in a FACScan. Surface expression of the (β_2-microglobulin-HLA-A*0201) construct was see after 12 hours with 1μM Ponasterone A, see Figure 35, panel B. Ponasterone A at 0.1μM induced little or undetectable amounts of the construct on the cell surface after 24 hours of culture in the presence of the inducer (Figure 35, panel D).

To examine the functional capability of the (β_2-microglobulin-HLA-A*0201) construct at the cell surface, a stimulated sample of sub-clone 9/20 was tested as targets in a cytotoxic T lymphocyte assay. The target cells were initially established as duplicate cultures of sub-clone 9/20 cells (together with duplicate cultures of untransfected CHO cells, to serve as negative controls). One of each duplicate culture was stimulated with Ponasterone A for 24 hours as described in Materials and Methods section 2.3.3. After labeling the cells with ^{51}Cr, half of each cell preparation was incubated with influenza A matrix peptide (GILGFVFTL) for 1 hour (see section 2.3.3), and half remained untreated for control purposes. Effector cells were the Human HLA-A2 restricted, influenza A matrix peptide specific CTL line, PG (Gotch et al., 1987). The CTL assay was performed as described in Materials and Methods section 2.3.3 using E:T ratios from 10:1 to 1.25:1. As can be seen from Figure 36 the stimulated, peptide pulsed sub-clone 9/20 cells acted as efficient targets for the PG effector cells, with approximately 50% lysis, at the highest E:T ratio. Control unstimulated sub-clone 9/20 cells (with or without peptide pulse) and stimulated non-peptide pulsed sub-clone 9/20 cells were not lysed by the effector PG cells. In addition, the control untransfected CHO cells, however treated, failed to be lysed in the assay by PG effector cells, see Figure 37.

FIGURE 33

Effect of the titre of Ponasterone A on (pIND-β_2M15-A2) construct expression in CHO clone 9/20 cells, analysed by ELISA using antibody L368.

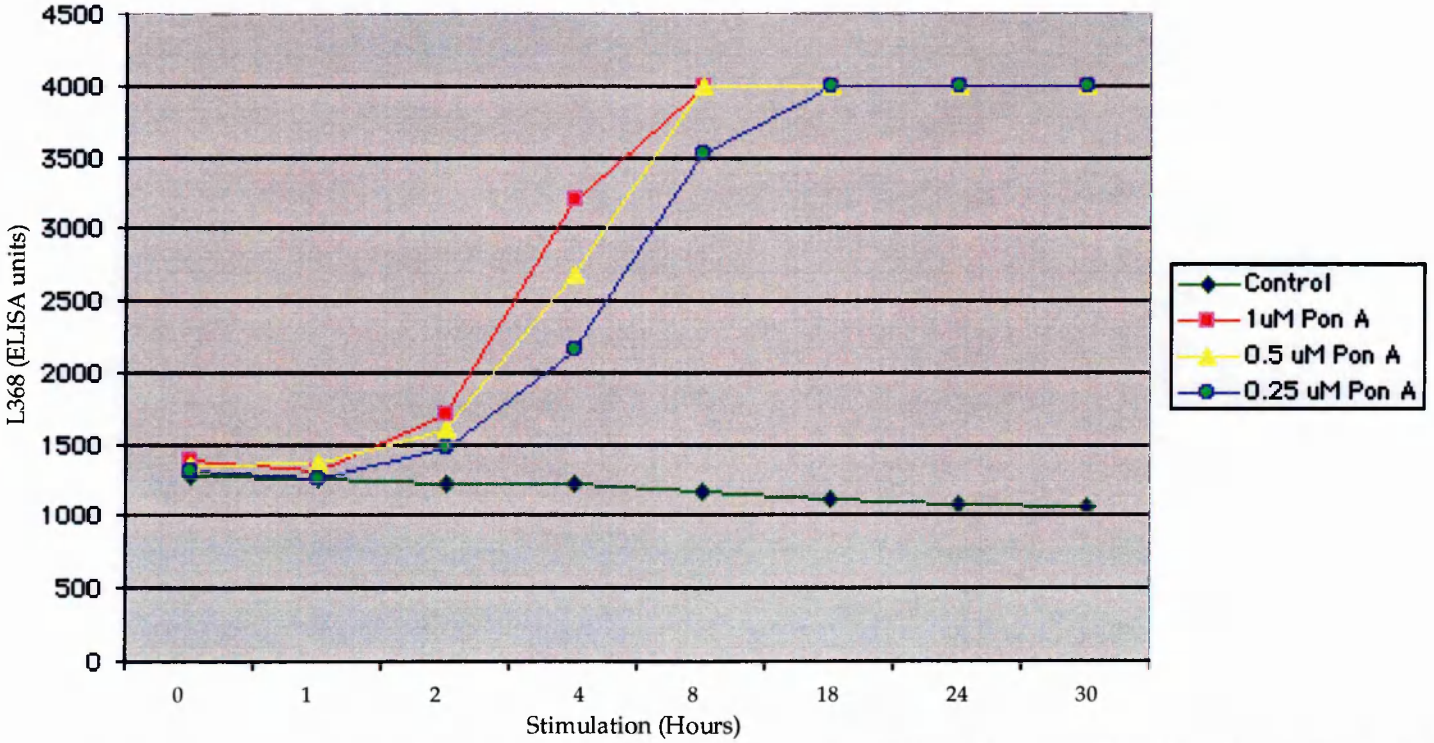


NB
4000 units is the maximum reading in the ELISA.

Individual cultures of CHO clone 9/20 cells were stimulated with increasing concentrations of stimulator, Ponasterone A, for 24 hours. Cells were analysed by ELISA using antibody L368 as described in Materials and Methods, section 2.3.3.

FIGURE 34

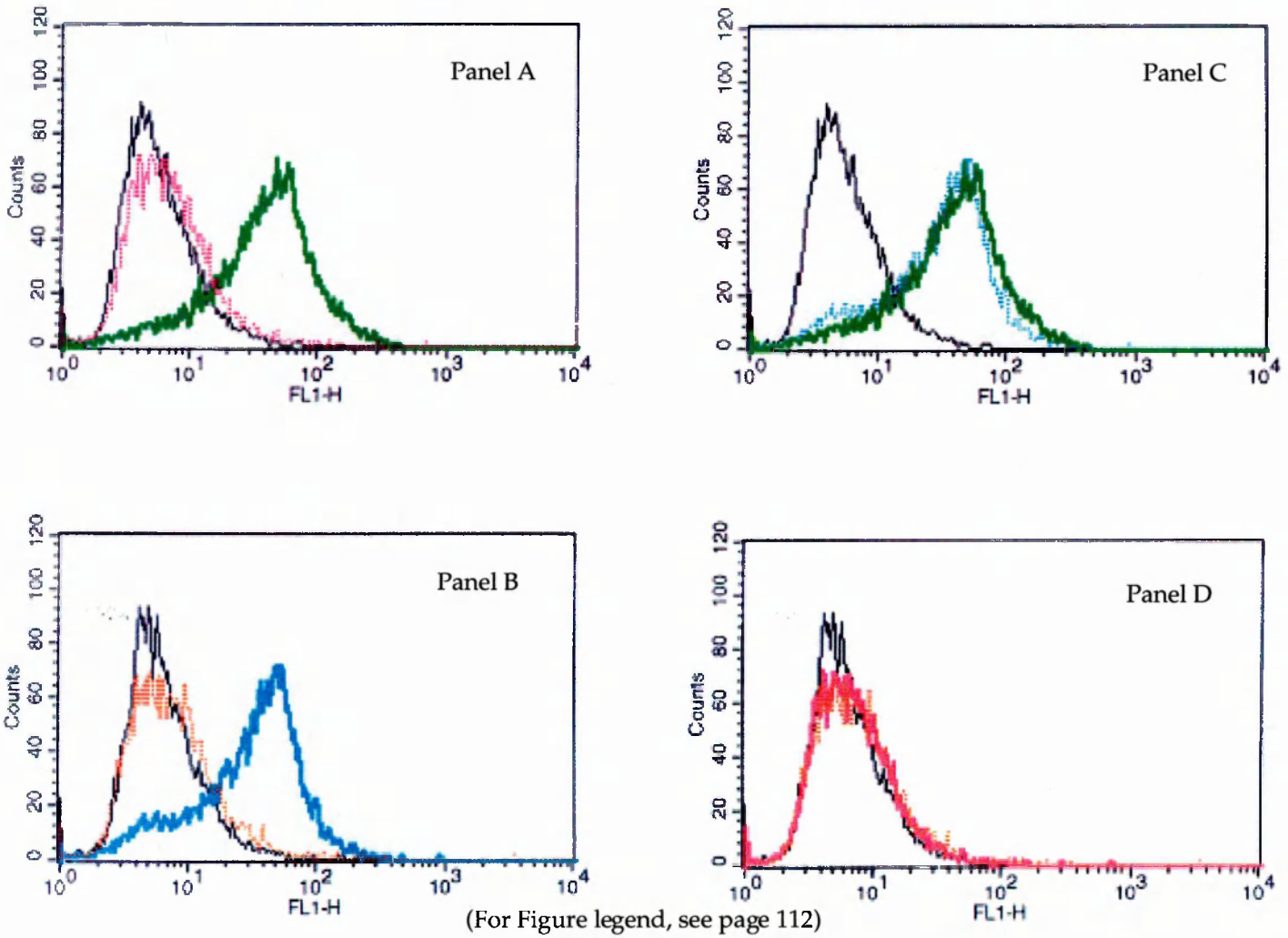
Time course of stimulation of (pIND-β_2M15-A2) construct expression in CHO clone 9/20 cells, Analysed by ELISA using antibody L368.



Individual cultures of CHO clone 9/20 cells were stimulated for different times, ranging from 0 to 30 hours with increasing concentrations (μM) of stimulator, Ponasterone A. Expression of the (pIND-β_2M15-A2) construct was monitored by ELISA using antibody L368 as described in Materials and Methods, section 2.3.3.

FIGURE 35

FACS analysis of the inducible expression of (pIND-β_2M15-A2) construct on CHO clone 9/20 cells.



(For Figure legend, see page 112)

Figure 35

FACS analysis of the inducible expression of (pINDβ_2M-A2)construct on CHO clone 9/20 cells.

Legend

Individual cultures of CHO clone 9/20 cells were stimulated for different times, either 12 or 24 hours, with two concentrations (0.1 and 1 μM) of stimulator, Ponasterone A. Expression of the (pINDβ_2M-A2)construct was then monitored by immunofluorescence as described in Materials and Methods, section 2.3.1. After staining the cells with primary antibody L368 , reactivity with this Antibody was detected using FITC conjugated goat anti-mouse. Analysis was then performed on FACScan.

Panel A: Clone 9/20 cells stimulated for 24 hours with 0.1μM (pink line) and 1μM (green line) Ponasterone A.

Panel B: Clone 9/90 cells stimulated for 12 hours with 0.1μM (orange line) and 1μM (blue line) Ponasterone A.

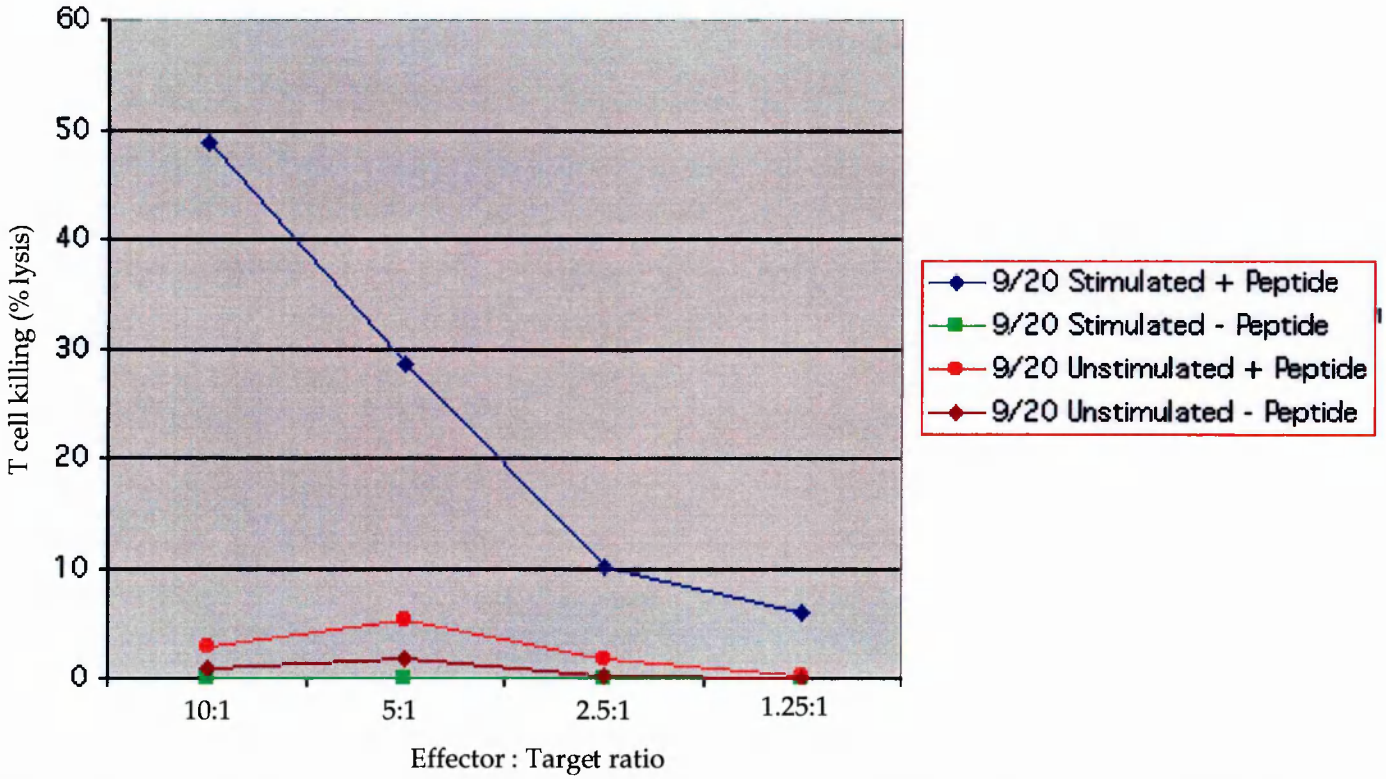
Panel C: Clone 9/20 cells stimulated with 1 μM Ponasterone A for 12 hours (blue line) and 24 hours (green line).

Panel D: Clone 9/20 cells stimulated with 0.1 μM Ponasterone A for 12 hours (orange line) and 24 hours (red line).

In each panel the negative control unstumulated cells are shown as the black line. These cells were not stimulated with Ponasterone A, but were analysed with antibody L368 and FITC goat anti-mouse.

FIGURE 36

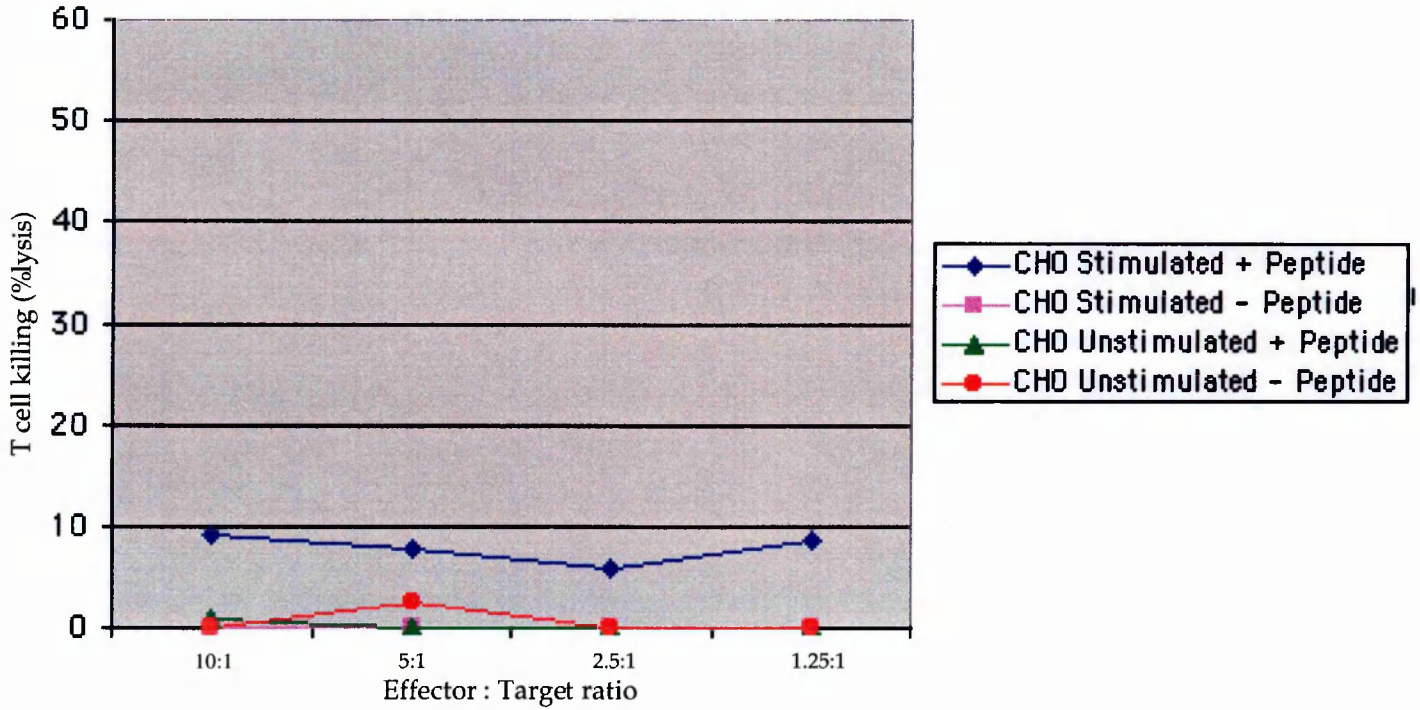
Cytotoxic T cell killing assay of CHO clone 9/20 cells induced to express the (pIND-β_2M15-A2) construct and pulsed with flu peptide.



Cultures of CHO clone 9/20 cells were either stimulated with Ponasterone A (1μM) for 24 hours, or unstimulated for control purposes,then pulsed for 1 hour with flu A matrix peptide (GILGFVFTL, 1μM), duplicates were incubated for 1 hour with culture medium only for controls, as described in Materials and Methods, section 2.3.3. All samples were labeled with ^{51}Cr and then used as target cells in a CTL assay using a Human HLA-A2 restricted, flu A matrix peptide specific cytotoxic T cell line, PG, as the effectors, with target ratios as shown. T cell killing was estimated as shown in section 2.3.3 and expressed as percent lysis of the target cells.
Samples were: i) 9/20 cells stimulated and pulsed with peptide (blue); ii) 9/20 cells stimulated but not pulsed with peptide (green); iii) 9/20 cells unstimulated, but pulsed with peptide (red) and iv) 9/20 cell unstimulated and not pulsed with peptide (maroon).

FIGURE 37

Cytotoxic T cell killing assay of untransfected CHO cells pulsed with flu peptide.



To serve as a control experiment, cultures of CHO were either stimulated with Ponasterone A (1μM) for 24 hours, or unstimulated for control purposes,then pulsed for 1 hour with flu A matrix peptide (GILGFVFTL, 1μM), duplicates were incubated for 1 hour with culture medium only for controls, as described in Materials and Methods, section 2.3.3. All samples were labeled with ^{51}Cr and then used as target cells in a CTL assay using a Human HLA-A2 restricted, flu A matrix peptide specific cytotoxic T cell line, PG, as the effectors, with target ratios as shown. T cell killing was estimated as shown in section 2.3.3 and expressed as percent lysis of the target cells.
Samples were: i) CHO cells stimulated and pulsed with peptide (blue); ii) CHO cells stimulated but not pulsed with peptide (pink); iii) CHOcells unstimulated, but pulsed with peptide (green) and iv) 9/20 cell unstimulated and not pulsed with peptide (red).

CHAPTER 4

DISCUSSION

4.1 Mutations in the β_2-microglobulin Gene.

At the time this thesis was started (1994) there were limited data on the frequency of loss of HLA class I expression in colorectal, and other tumours. Furthermore, most of the available information was derived from immunocytochemical analysis using monomorphic, and later polymorphic, antibodies. The exception was the Burkitts lymphoma cell line, Daudi, in which Goodfellow *et al.* suggested the lack of HLA class I expression in human-mouse hybrids was due to a loss of β_2-microglobulin caused by a genetic fault on chromosome 15 (Goodfellow et al., 1975a). Later, this genetic fault was shown to be a mutation in the initiation codon of the β_2-microglobulin gene, and coupled with LOH, led to the loss of β_2-microglobulin and hence the loss of HLA class I expression (Rosa et al., 1983). The colorectal cell line, LoVo, was also shown to lack β_2-microglobulin expression, leading to the absence of HLA class I on the cell surface, but the mechanism behind this loss had not been explained (Brodsky et al., 1979b; Travers et al., 1982).

In this thesis, therefore, the starting point was to devise an assay for screening cells for mutations in the β_2-microglobulin gene. The approach used was to amplify by PCR, approximately 300 bp regions of genomic DNA, followed by

analysis with the SSCP technique and then sequencing any candidates identified by SSCP. In the initial study of 52 colorectal cell lines, 8 were identified with mutations in the β_2-microglobulin gene, giving a frequency of 15.4% in the cell lines. If cell line pairs are taken into account (DLD-1/HCT-15 and GP2d/GP5d are pairs of cell lines from the same patients), 6 tumours from a total of 43 patients (see Table 4) show β_2-microglobulin mutations, representing a frequency of 14% in the colorectal tumour derived cell lines. In two large studies (99 and 70 cases) of colorectal tumours, using immunocytochemistry, reduced or total loss of HLA class I was reported in 17% and 23% of tumours respectively (Kaklamanis et al., 1992; Lopez-Nevot et al., 1989). These studies took into account both total and reduced levels of HLA class I and losses of individual HLA class I alleles, therefore, the lower incidence of mutations in β_2-microglobulin, in this study, is in keeping with these data.

In the study of 280 fresh tumours, including colorectal, melanoma, breast, ovary and lymphoma (see Table 7) ten mutations in β_2-microglobulin were found by SSCP (9/147 colorectal carcinomas and 1/133 non-colorectal tumours). Thus, among the tumours analyzed, β_2-microglobulin mutations were significantly ($p<0.02$) overrepresented in the colorectal carcinomas. In all of the fresh tumour DNA samples, both the wild-type and mutated β_2-microglobulin alleles were present. This may indicate heterozygosity for β_2-microglobulin mutations or might reflect contamination with normal tissue. However, 7 of the 9 fresh tumour samples identified with β_2-microglobulin mutations were also examined by immunocytochemistry. In three of these samples the tumour cells showed no reactivity with antibodies W6/32 and BBM.1, see Table 9, indicating that these tumours were probably homozygous for their respective β_2-microglobulin mutations, but the others were presumably heterozygous.

The identification of defects in DNA mismatch repair (or mutator phenotype) has been linked with microsatellite instability. In the panel of 40 colorectal cell lines, 10 demonstrated microsatellite instability. Seven of these contained mutations in the β_2-microglobulin gene (see Table 10, HRA-19 was not analyzed for microsatellite instability). Three lines exhibited microsatellite instability but did

not have mutations in β_2-microglobulin. However, one of these, HCA-7, has been reported not to express HLA-A1, but a normal lymphoblastoid B cell line from the same patient expressed HLA-A1 (Smith, 1989) and LS174T lacks expression of HLA-A2. Both the colorectal cell lines HCA-7 and LS 174T type positively, at the DNA level, for the respective alleles that are not expressed (Browning et al., 1996). A third colorectal cell line, LS411, shows loss of expression of HLA-A2 and HLA-A11, when compared to a corresponding normal lymphoblastoid B cell line, RN, so that in this case two genetic events have occurred. LS411, when typed at the DNA level, showed the absence of an HLA-A11 gene, indicating gene loss perhaps due to LOH, but the HLA-A2 gene was present. However, when LS411 was analyzed with antibody BB7.2 (recognizing HLA-A2, -A69) in an ELISA assay, no reactivity was seen, suggesting selective loss of expression of HLA-A2 (Browning et al., 1993).

In the study of fresh colorectal tumours, 71 were analyzed for microsatellite instability and 7 (10%) showed evidence of alterations at two or more microsatellite loci and were judged to be unstable. These observations are consistent with an earlier study, reporting 10-15% of sporadic colorectal carcinomas exhibiting microsatellite instability (Peltomaki et al., 1993). No instability was detected among the normal DNA samples (<3%). Instability was observed in five of the nine tumours with β_2-microglobulin mutations, see Table 10. These include tumours that are homozygous or heterozygous for β_2-microglobulin loss, as well as apparent heterozygotes in which β_2-microglobulin expression was considerably reduced, see Table 9. Mismatch repair defects are therefore significantly more common amongst tumours with β_2-microglobulin mutations (5/9) than in colorectal carcinomas in general ($p<0.01$). Microsatellite instability has been reported to be infrequent in melanoma (Quinn et al., 1995) and breast cancer (Yee et al., 1994) and is consistent with the much lower frequency of β_2-microglobulin mutations (1/133) found in these tumours, and other carcinoma types, in the study.

If the data from colorectal cell lines and fresh tumour studies are combined, then 16 mutations were identified in 15 individual tumours. (Several pairs of cell lines

were used in the study and some samples exhibited two different mutations.) Ten mutations were seen in the Leader peptide/exon 1 region, 7 of which occurred in the same 8 bp $(CT)_4$ repeat sequence. Five of the seven were microsatellite unstable. One of the seven (tumour C14) was microsatellite stable and (HRA-19) was not analyzed for instability. It does appears, therefore, that this 8 bp $(CT)_4$ repeat sequence is particularly vulnerable to errors in DNA replication, especially slippage of 2 or 4 bp, and is not efficiently repaired in cells showing microsatellite instability. Instability at microsatellite sites is a recognized marker for mismatch repair-defects. An increased mutation rate alone is unlikely to account for the observed frequency of β_2-microglobulin mutations in mismatch repair-defective colorectal tumours. Of 16 mutations identified in the β_2-microglobulin gene, from 15 tumours, at least four did not demonstrate microsatellite instability (the cell line HRA-19 was not analyzed for instability). The high frequency of β_2-microglobulin mutations among tumours with a normal spontaneous mutation rate implies the contribution of a second factor influencing the emergence of tumours with mutations in β_2-microglobulin. In both the colorectal cell lines and fresh tumours studied, the mutation was associated with a significant reduction in expression of β_2-microglobulin (see Tables 6 and 9). The selective advantage gained by consequent loss of expression of HLA class I molecules at the cell surface with the loss in ability to present HLA-associated antigens is therefore the most likely contributing factor. The primary role of HLA class I/β_2-microglobulin complexes, in immune surveillance, is the presentation of peptide antigens to circulating cytotoxic T lymphocytes and the loss of this role would give the tumour a distinct survival advantage from T cell attack. Not all the tumours in the study with β_2-microglobulin abnormalities exhibit complete loss of β_2-microglobulin expression and several cases of heterozygous mutations were identified. These tumours appear to express intermediate levels of HLA class I (see Tables 6 and 9) which in itself might be a selective advantage to these tumours by limiting the efficiency of peptide presentation. This could be particularly relevant in tumours exhibiting microsatellite instability where increased numbers of potential peptide antigens are generated.

4.2 Mutations in specific HLA class I alleles.

The colorectal cell line HCA-7 has been of some intrigue since the report by Mark Smith that it lacked expression of HLA-A1, but that a corresponding normal B lymphoblastoid cell line, EVA-1224, derived from the same patient, clearly expressed this allele (Smith, 1990). He indicated that data from both ELISA and immunocytochemical assays, using antibody Mab142.2 (which recognizes HLA-A1 [Dr. S. Radka and Genetic Systems]), supported the suggestion that the colorectal cell line had selectively lost expression of HLA-A1. Stimulation with γIFN increased HLA-A2 expression in both cell lines, but after stimulation for 24 hours, the expression of HLA-A1 could not be re-established on HCA-7 cells. Later, when an HLA-A locus typing system had been developed, based on typing the DNA using allele specific primers in PCR, Browning et al showed that both HCA-7 and EVA-1224 typed for HLA-A1 and -A2 and suggested that the loss of HLA-A1 expression in HCA-7 was due to some other mechanism than gene loss (Browning et al., 1993). The HLA-A locus typing of these two cell lines was repeated using the AMDI technique, with additional data for HLA-B and C loci showing identical typing for both cell lines, confirming they are derived from the same individual (see Figure 13A&B).

In this thesis, the genetic event leading to the loss of HLA-A*0101 expression in HCA-7 has been identified as an insertion of an additional cytidine residue into a run of 7 cytidines (bp 621-627), located at the beginning of exon 4 (Figure 18 and Table 11). Investigation of the mRNA, using a series of allele specific primers for the analysis, indicated that a short message for HLA-A*0101 was seen in HCA-7, compared to an apparent full length in EVA-1224. (The primers used for this assay covered the HLA-A*0101 gene from the middle of exon 2, at the 5′ end, to beyond exon 8 in the untranslated region, 3′ to exon 8 [see Figure 15].) In HCA-7 the message is probably the length of the first three exons of HLA-A*0101, because with primers AL-16 and AL-x, a RT-PCR product was present, but with primers AL-16 and 61998,46992 and HLA3UTA no amplicon was seen in the RT-PCR, as shown in Fig 16. In exon 4 the $(C)_7$ island is next to a (GA) which contributes to the splice site between exons 3 and 4. It is therefore plausible that

the insertion of an additional cytidine into this $(C)_7$ run will have a negative influence on the splice site formation, leading to a short message being translated. By comparison both cell lines appear to contain full-length messages for HLA-A*0201.

The insertion of a cytidine into the $(C)_7$ sequence, at the beginning of exon 4 of HLA-A*0104, has been noted (Laforet et al., 1997) and the individuals carrying this HLA-A*0104"null" gene lack expression of the allele product on the surface of their cells. A similar insertion of a single cytidine into this $(C)_7$ island has also been reported for HLA-A*2411"null" (Magor et al., 1997). In these cases the cytidine insertion has been suggested to be the result of slipped-strand mispairing (Parham, 1997). The finding here, in the colorectal carcinoma cell line HCA-7, of a cytidine insertion into the $(C)_7$ sequence is more likely to be the result of an un-repaired mismatch, that occurred during DNA replication, since the cell line has been reported to carry the mutator phenotype (Branch et al., 1995). Although HCA-7 appears to have the HLA-A*0101"null" allele, the corresponding normal B cell, EVA-1224, clearly types as HLA-A*0101. Therefore, strictly speaking, HCA-7 carries a mutated HLA-A*0101 gene with the same sequence as that proposed for the HLA-A*0101"null" allele.

The cytidine repeat sequence at the beginning of exon 4 in the HLA class I is interesting when looking at the different alleles. The HLA-A* loci fall into two groups in this region, HLA-A*01, *03, *11, *23, *24, *30, *31, *36, *80 and *3303 alleles have the $(C)_7$ sequence in this region, whilst most other HLA-A* alleles have a $(C)_5$ repeat at this point. HLA-B* alleles have 5, 6 or 7 (C) repeats and some of the *C alleles only have 4 repeated (C)'s. The risk, therefore, of a "null" allele being created may be hierarchical amongst the HLA alleles according to the number of cytidines in this region (Bunce et al., 1999). If this is true for the appearance of "null" alleles, then there is the possibility that, especially in mutator phenotype positive tumours, this tendency will be the same and HLA-A alleles may be mutated more frequently than HLA-C alleles.

4.3 Re-expression of HLA and β_2-microglobulin.

Many defects in the expression of HLA and other molecules involved in the HLA pathway have been reported in tumours and these defects have been attributed to selection for escape from T cell immune attack (Bodmer et al., 1993). Such changes include transporter defects leading to the loss of effective antigen processing as described in the cell lines RMA-S and .174T (Cerundolo et al., 1990; Townsend et al., 1989). Several early attempts to express MHC constructs have been reported. Mottez et al. were able to express a mouse K^d/β_2-microglobulin construct in monkey COS-1 cells and demonstrate surface expression by K^d specific antibodies and the binding of HIV viral peptides that had previously been shown to bind strongly to native K^d (Mottez et al., 1991). Similarly, surface expression of a mouse H-2D$^d/\beta_2$-microglobulin was shown on KJ29 human kidney carcinoma cells after transfection of a single gene construct. In this case the product of this construct was demonstrated to be capable of presenting an HIV gp100 peptide, p18I10, by pulsing the transfetced cells with peptide, then showing specific cell lysis by the H-2D^d restricted T cell hybridoma, B4.2.3 (Lee et al., 1994) .

The human colorectal cell line, DLD-1, was known to completely lack expression of HLA-A, B and C genes, although at the time, the underlying genetic defects leading to this loss of expression were unknown (Smith, 1990). This cell line has been useful in constructing models for re-expressing HLA molecules and for studying the functional contribution of other transporters, proteasomes and associated molecules in the pathway of antigen presentation by the HLA system. Studies using DLD-1 and a single chain HLA-A*0201/β_2-microglobulin construct showed surface expression of the construct with functional capabilities. This was demonstrated in two ways, first by exogenously pulsing the transfected DLD-1 cells with a flu matrix 1 peptide antigen (M58-66), followed by specific lysis in a CTL assay using a T cell clone which recognized the flu antigen in an HLA-A2 restricted manner. Additional functional evidence was shown by endogenously infecting the tranfected DLD-1 cells with the recombinant vaccinia (M1-Vac) product and showing specific lysis in a CTL assay using the same T cell clone.

However, the CTL recognition was only detectable after stimulation of the DLD-1 cells with IFN-γ, which was suggested indicated an underlying defect, other than the loss of HLA expression, in the antigen processing pathway (Toshitani et al., 1996).

These experiments introducing HLA molecules into cells that normally do not express them, or re-expressing HLA molecules in tumour cells that have lost HLA expression due to a specific gene mutation, have been useful for understanding some aspects of the peptide antigen presentation pathway. However, in these experiments negative controls were always a compromise, being either untransfected cells or cells transfected with the empty plasmid vector (Lee et al., 1994; Toshitani et al., 1996). With the advent of the Ecdysone Inducible Expression System, transfected cells constitutively express the DNA binding proteins RxR and VgEcR, but only express the gene of interest in the presence of an inducer, Ponasterone A (see Figure 5). In experiments using this system, the control cells take the form of transfected cells that are not exposed to the inducing agent (No et al., 1996).

Re-expression of HLA class I on the colorectal cell line, DLD-1, was attempted using the Ecdysone Inducible Expression system with two constructs, one for β_2-microglobulin (pIND-β_2M) and one for a single chain β_2-microglobulin-$(GGGGS)_3$linker-HLA-A*0201 (pIND-β_2M-15A2). Initially, individual clones were isolated and analyzed by ELISA. Two clones from each transfection were selected, see section 3.4.2, and bulk cultures grown. Further selection of cells responsive to the inducer, Ponasterone A, was attempted using magnetic bead technology. After four rounds of magnetic bead selection, it appeared, as shown in Table 12, that the number of cells positively staining with antibody L368 (anti-β_2-microglobulin) had increased in each clone with additional rounds of the bead selection. However, it became apparent that the cells positively selected were, in fact, not responsive to the inducer, and had integrated the (pIND-β_2M) and (pIND-β_2M-15A2) plasmids in such a way that the expression of the constructs was permanently on. This was shown by growing representative samples of the bead-sorted clones in microtitre plates and stimulating with Ponasterone A and assaying by ELISA as performed in the initial analysis following transfection.

Table 13 shows that after 4 bead sorts and culturing for approximately 4 weeks (4 passages) and 7 weeks (7 passages) the unstimulated control cells expressed the respective constructs as strongly, by reactivity with antibody L368, as those cells exposed to the inducer. At this stage attempts were made to clone these 'permanently on' cultures by limiting dilution to try and find a sub-clone that was responsive to the inducer. This was not successful (data not shown) and after screening over 500 sub-clones the attempt was abandoned. A similar cloning by limiting dilution was performed with examples of the primary clones isolated post transfection and not bead sorted, but again no sub-clones responsive to the inducer were identified.

Following these unsuccessful attempts to express the single chain (β_2M-15A2) and (β_2M) constructs in DLD-1 colorectal cells, using the Ecdysone inducible expression system, a second attempt was made to establish this system in CHO.K1 Chinese hamster ovary cells. This line was chosen because of its ease to grow in culture and transfect, and additionally did not react with the antibodies L368 and W6/32. Using CHO cells, successful transfections of both (pIND-β_2M-15A2) and (pIND-β_2M) plasmids was achieved and the initial clones isolated appeared to be responsive to the inducer, Ponasterone A. Following cloning by limiting dilution, two sub-clones from each of the (pIND-β_2M-15A2) and (pIND-β_2M) transfections were identified and these demonstrated expression of the respective constructs to be under the control of the inducer, see Figures 26-29. Further analysis of these clones revealed that the stimulated (pIND-β_2M) clones reacted positively with antibody L368 in ELISA, but were negative when analyzed by immunofluorescence and FACscan. This indicated that these sub-clones, although expressing $\beta_2$microglobulin in the cytoplasm, were not expressing it on the cell surface. One of the (pIND-β_2M-15A2) sub-clones, number 9/20, however, showed expression of the construct on the cell surface and that this expression was under the control of the inducer. In addition, expression in this sub-clone was shown to be under the influence of both concentration and length of time of stimulation by the inducer, see Figures 33 and 34.

Cells from the (pIND-β_2M-15A2) sub-clone 9/20 were stimulated to express the construct at the cell surface and were then pulsed with influenza A matrix peptide. Recognition of these cells was then achieved in a cytotoxic T cell assay involving an HLA-A*2-restricted $CD8^+$ T cell clone raised to the flu A matrix peptide. Transfected, unstimulated sub-clone 9/20 cells, also pulsed with flu peptide were not recognized in this assay, see Figures 36 and 37.

Similar cytotoxic T cell recognition has been reported with the colorectal cancer cell line, DLD-1, stably transfected with the (pcDNA-β_2M-15A2) plasmid and pulsed with the flu matrix peptide (Toshitani et al., 1996). In addition, the DLD-1 transfectant, after treatment with γIFN, was able to present antigen (influenza matrix 1 protein, M1-Vac) that had been introduced into cells by infection with recombinant vaccinia virus. This study extends the data by expressing the β_2M-15A2 construct in an inducible expression system and shows that the construct maintains a functional capacity. In earlier work wild type $\beta_2$microglobulin was introduced into DLD-1 cells by infection with recombinant vaccinia virus encoding the human $\beta_2$microglobulin gene. In this case only marginal re-expression of $\beta_2$microglobulin and HLA-A*2 was seen. If the DLD-1 cells were pre-treated with γIFN before infection, HLA class I was restored (Browning et al., 1996). Such a finding was suggested to indicate that the DLD-1 cells also contained another defect in HLA class I expression and that defect might be in the TAP processing of antigen, since a similar defect in HLA class I expression had been reported in the cell line T2 (Salter and Cresswell, 1986). Peptide expression by HLA class I has now been achieved using a single chain construct of peptide linked to the 5′ end of $\beta_2$microglobulin with a $(GGGS)_3$ spacer and the 3′ end of $\beta_2$microglobulin linked by a second $(GGGS)_3$ spacer to the α1 domain of HLA-A*2 (Greten et al., 2002). In these experiments $CD8^+$ T cells, raised against the peptide, were shown to recognize COS cells transiently expressing the single chain product. This single chain construct has also been linked with a third $(GGGS)_3$ spacer placed between the α3 domain of HLA-A*2 and an IgG1 heavy chain sequence. This complex has been expressed in J558L cells and the resulting

proteins used to form multivalent complexes that have been used to isolate peptide specific $CD8^+$ T cells from human peripheral blood.

4.4 Concluding Remarks.

Loss of $\beta_2$microglobulin as an underlying cause for lack of all HLA class I expression has been reported in studies based on immunocytochemical analysis of colorectal tumours (Momburg and Koch, 1989; Rees et al., 1988; Smith, 1989) and colorectal cell lines (Arce-Gomez et al., 1978). The present study identifies some of the genetic events that lead to the failure of $\beta_2$microglobulin expression. Loss of $\beta_2$microglobulin expression has profound effects on peptide presentation to cytotoxic T cells because of the requirement of all HLA class I molecules to combine with $\beta_2$microglobulin for effective antigen presentation. The data here also indicates that loss or reduced $\beta_2$microglobulin expression can arise through mutation in the $\beta_2$microglobulin gene, and that this event is particularly likely in colorectal tumour cells with defects in DNA mismatch repair. These tumour cells are potentially prime targets for cytotoxic T cells because they will express many neoantigens derived from genetically altered proteins. Tumour cells that survive this immune surveillance may have had to undergo a particularly severe selective pressure to escape T cell killing through the loss of HLA class I expression.

Loss of HLA class I expression may also result from malfunction of other molecules involved in the HLA class I assembly pathway. Failure to identify the presence of TAP1 correlated with a lack of HLA class I expression at the cell surface in colorectal cancers (Kaklamanis et al., 1994) and cervical carcinomas (Cromme et al., 1994). Loss of tapasin has been investigated in the .220 B cell line and although HLA class I molecules have been detected on the cell surface they appear to be only poorly expressing a limited number of peptides, indicating that tapasin is crucial in peptide binding to HLA class I-$\beta_2$microglobulin moieties (Grandea et al., 1995). Lack of expression of calnexin, however, appears not to affect the expression of HLA class I-$\beta_2$microglobulin and normal presentation of

peptides was seen in the calnexin deficient cell line CEM-NKR (Scott and Dawson, 1995).

The loss of expression on tumours of a complete HLA class I haplotype, as a means of escaping immune surveillance, has been reported (Smith, 1989). Complete HLA class I haplotype loss was also seen in several colorectal cancer cell lines that were typed by PCR, at the DNA level (Browning et al., 1996). The underlying mechanisms suggested for these cases were chromosomal nondisjunction or mitotic recombination. Monoclonal antibodies with polymorphic reactivity have been used to record the loss of expression of individual HLA class I alleles in colon carinomas (Brodsky et al., 1979b; Kaklamanis et al., 1992; Smith, 1989). Different alleles can present different antigenic epitopes to cytotoxic T cells and therefore selective loss an individual HLA class I allele will have the consequences of reducing the peptide repertoire presented. A tumour expressing an epitope that elicitates a particularly severe cyotoxic T cell response would be under strong selective pressure to loose that expression which could be achieved by the loss of the relevant HLA class I allele. It has been suggested that this is evidence of immune selection (Brodsky et al., 1979b).

Many support the hypothesis that loss of HLA class I expression can lead to the failure of tumour cells to present peptide epitopes to $CD8^+$ cytotoxic T cells (Arce-Gomez et al., 1978; Bodmer, 1987; Branch et al., 1995), but this brings about a paradox, in that cells lacking expression of HLA class I are liable to attack from NK cells, whereas tumours appear to escape this form of immune surveillance. The NK cells express a series of natural cytotoxic receptors (NCR), including 2B4 and NKp80, which bind to ligands on tumour cells. These and other coreceptor interactions provide the 'on' signal for NK activity, but in normal cells an 'off' signal from the HLA-class I-specific inhibitory receptor appears to be stronger, when it interacts with HLA class I, preventing serious cell damage. In tumour cells lacking HLA class I, the 'on' signal would be expected to dominate. However, in these tumours other unknown ligands may interact with NK receptors involved in the NK cell-target recognition, and in the case of some

tumours, these are still affective interactions providing an 'off' signal to the NK cells preventing tumour lysis (Moretta et al., 2001).

The clinical importance of HLA class I loss in colorectal carcinomas is largely unknown. There is no correlation between HLA class I loss and Dukes staging, which is still the most widely used prognostic indicator used in practice. One study, looking at the overall expression of HLA class I with antibody W6/32, found no correlation post-operatively between tumour recurrence and HLA class I expression on the primary tumour (Moller et al., 1991). However, there is some evidence to suggest that tumours that have lost HLA class I expression are likely to have a poorer prognosis (Bodmer, 1987). These tumours may be more "aggressive" and as they progress are more likely to acquire changes on their cell surface, in the form of tumour antigens, that could be recognized by cells of the immune surveillance system. As a result these tumours would be subjected to stronger selection by the development of immunity to cytotoxic T cell attack through reduced or lost expression of HLA class I and antigen presentation. However, in a recent study of colorectal tumours reduced expression of HLA class I was seen in 63% and complete loss in 7% (Menon et al., 2002) . Reduced or absent HLA class I expression correlated with a lower tumour stage and longer disease-free survival. In this study most of the loss of HLA class I expression was attributed to mutations in $\beta_2$microglobulin and half of these mutations occurred in tumours exhibiting a mutator phenotype. The suggestion was made that the longer survival could be due to elimination of HLA class I negative cells by natural killer cells or by an attenuated tumour aggressiveness often seen in tumours with a mutator phenotype.

The mutator phenotype, characterized by DNA mismatch repair defects, combined with a selective pressure have been associated with the overrepresentation of mutations in the transforming growth factor-β receptor type II (TGF-β receptor II) in colorectal tumours (Markowitz et al., 1995). This gene also contains a series of repeated sequences that are subject to frameshift mutations in microsatellite unstable colorectal tumours, leading to the inactivation of the TGF-β receptor II. Endometrial tumours are not normally responsive to the TGF-β factor and mutations in the TGF-β receptor II have rarely

been found in these tumours (Myeroff et al., 1995). Thus the acquisition, in the colon carcinomas, of a mutation in a specific hotspot, in the sequence of TGF-β receptor II, and selective pressure were both required for the development of tumours with an altered genotype.

The loss of HLA class I expression, whether it be total loss due to mutations in $\beta_2$microglobulin or loss of a single HLA class I allele, appears to compromise the presentation of peptide epitopes to cytotoxic T cells. The results shown here demonstrate that these losses of HLA class I expression can be explained by genetic events both in the $\beta_2$microglobulin gene and in a specific HLA class I allele. Re-expression of HLA class I has been acheived by transfecting a human $\beta_2$microglobulin-HLA class I single chain construct into the CHO hamster cell line, using an inducible expression system, and the expression of the construct demonstrated to be controlled by an inducer, Ponasterone A. The transfected stimulated CHO cells were capable of presenting an influenza A matrix peptide antigen, in an HLA-A2 restricted manner, to a human cytotoxic T cell clone. CHO cells expressing the $\beta_2$microglobulin-HLA class I single chain construct, using the inducible expression system, should be useful in the future for studying the pathway of peptide antigen presentation. The system could be used to pulse the transfected stimulated CHO cells with different peptide antigens and to investigate the efficiency of presentation, in this caes, by HLA-A2. Furthermore, the CHO cells could be infected with viral antigens and upon stimulation with Ponasterone A, used to study peptide antigen processing involving proteasome and transporter molecules in addition to the $\beta_2$microglobulin/HLA complex. Such studies may be useful in the development of anti-cancer vaccines.

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